JOURNAL OF THE NATIONAL CANCER INSTITUTE

July 1956
Volume 17 • Number 1
A Publication of the
U. S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
Public Health Service
National Institutes of Health

JOURNAL OF THE NATIONAL CANCER INSTITUTE

JOHN R. HELLER, *Director*
National Cancer Institute

BOARD OF EDITORS

MICHAEL B. SHIMKIN, *Scientific Editor*

W. RAY BRYAN, *Associate Editor*
ELBERT A. PETERSON, *Associate Editor*
ELI M. NADEL, *Associate Editor*
EMMA SHELTON, *Associate Editor*
SEYMOUR H. WOLLMAN, *Associate Editor*

EDITORIAL STAFF

DORIS M. CHANEY, *Managing Editor*

ISABEL K. NEELY, *Asst. Managing Editor*
RENÉE T. COCHIN, *Assistant Editor*

U. S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NATIONAL INSTITUTES OF HEALTH
NATIONAL CANCER INSTITUTE
BETHESDA 14, MARYLAND
UNITED STATES OF AMERICA

UNIVE
OF MICHIGAN
AUG 21 1956
MEDICAL

JOURNAL OF THE NATIONAL CANCER INSTITUTE

NATIONAL INSTITUTES OF HEALTH • Public Health Service

Volume 17
Number 1

July 1956

ANNOUNCEMENT

The *Journal of the National Cancer Institute* will be published monthly starting with the current issue, Volume 17, Number 1, July, 1956. Thereafter, two volumes of six issues each will appear, beginning with Number 1 in July and January of every year.

This change of schedule, after 16 years of bimonthly publication, has been made to increase the publication outlet for investigators engaged in cancer research. Papers on basic research, clinical investigations, statistical studies, and critical reviews in cancer are invited.

The Journal may be purchased from the Superintendent of Documents, U. S. Government Printing Office, Washington 25, D. C. Subscription prices per year for two volumes are as follows: United States, Canada, and Mexico—$20.00; Foreign—$25.00; Single issues—$2.00 each.

◆ ◆ ◆

Experiments on an Ascites Hepatoma. III. The Conversion of Mouse Hepatomas into the Ascites Form[1]

HARUO SATO,[2,3] MORRIS BELKIN, *and* EDWARD ESSNER, *Laboratory of Chemical Pharmacology, National Cancer Institute,*[4] *Bethesda, Maryland*

Malignant cells differ widely in their ability to survive when suspended in the fluid of the peritoneal cavity. Indeed, the production of a true ascites tumor is by no means always a simple task. Thus, for example, Klein (*1,2*) could convert only 12 out of 42 solid tumors into the ascites form. Six tumors were converted immediately and 6 others gradually, by successive transfers of the peritoneal exudate, while the remaining 30 tumors failed to transform into ascites tumors. Similarly, Yoshida (*3*), in reviewing the work of the Japanese group on the ascites hepatoma of the rat, indicated that of a total of 61 histologically malignant hepatomas, 56 failed to "take" upon intraperitoneal transplantation. Of the five tumor "takes" only three were immediately convertible into the ascites form, and further attempts yielded only two successful ascites hepatomas.

The question as to why some tumors convert immediately into an ascites form, while others do so only gradually or not at all, is in turn related to fundamental problems of selection and adaptation and to the degree to which tumor cells in a given population differ physiologically from one another. Thus far, the results of Klein indicate that the ability of a tumor to convert into an ascites form is due in part to a rapid growth rate and/or high degree of anaplasia and, what is perhaps more important, to a process of selection of cells possessing a predilection for growth in the ascites form (*4,5*).

The question of the possible relation between anaplasia and the production of ascites tumors was also raised by Sato and Aruji (*6*) and by Aruji (*7*) in their studies on the ascites hepatoma of the rat, but no clear-cut correlations appeared to exist.

In the present study, the role of anaplasia as a factor in ascites-tumor formation was investigated in hepatomas of the mouse, most of which had undergone relatively few animal passages. A description of two solid mouse hepatomas successfully established as ascites tumors, and two ascites-tumor sublines which arose by an interesting alteration in one of

[1] Received for publication February 1, 1956.
[2] Visiting Scientist, National Cancer Institute.
[3] *Present address:* Department of Pathology, Fukushima Medical College, Fukushima City, Japan.
[4] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

these tumor ascites, is presented. The histology of the original tumors and of the ascites-tumor lines reconverted into solid form, the distribution of the chromosome number, and survival curves of the tumor-bearing mice are presented. Based upon these data, the problem of the relation of anaplasia to the ability of tumors to grow in the ascites form is discussed.

Material and Methods

Each of the 16 mouse hepatomas [5] used in the present study was induced in C3H mice by administration of carbon tetrachloride or 4-*o*-tolylazo-*o*-toluidine [6] and then carried subcutaneously through several generations prior to intraperitoneal inoculation. The technique for conversion of these solid hepatomas into ascites tumors was essentially similar to that described for rat hepatomas (*6*). The solid tumor was removed under sterile conditions and forced through a tissue press containing a metal disk in the form of a fine sieve, and the resulting mash was suspended in physiologic saline. About 0.1 to 0.2 cc. of tumor mash was then inoculated intraperitoneally into mice (C3H/HeN) weighing about 20 gm.

Routine transplantation of the tumor ascites and withdrawal of ascites for purposes of examination were performed with the use of sterile glass pipettes as described previously (*8*).

Sections of solid tumors and of other tissues were fixed in Zenker-formol and stained with hematoxylin and eosin. Samples of the free-cell tumor ascites were examined on air-dried, Giemsa-stained smears. Samples of tumor ascites containing islands were mixed with an equal volume of 1 to 2 percent aceto-orcein (dissolved in 50% acetic acid) and stored in glass vials at room temperature. For chromosome study, the tumor cells and tumor islands were pretreated by immersion in 0.5 percent NaCl for 15 minutes prior to the addition of aceto-orcein.

Results

The results of the present series of experiments on the conversion of solid hepatomas into the ascitic form are shown in table 1. Hepatomas 134 and 129 (in group A) were converted into island ascites tumors in the first intraperitoneal transplantation. Hepatomas 129(P) and 129(F), which are free-cell ascites hepatomas, are two sublines derived from hepatoma 129 after its establishment as an island ascites tumor. In five other hepatomas (group B) intraperitoneal inoculation resulted not in ascites-tumor growth but only in the production of solid-tumor tissue together with a quantity of ascitic fluid. In some cases this ascitic fluid contained a small number of tumor islands but, while further intraperitoneal transplantation (2 to 4 generations) continued to stimulate exudate formation in the inoculated mice, this did not result in the formation of ascites tumors. In seven other hepatomas (group C), the tumors grew intraperitoneally only in solid form and failed to convert into ascites tumors. Two tumors of

[5] We wish to thank Dr. H. B. Andervont and Dr. A. J. Dalton for supplying the mouse hepatomas used in this study.

[6] Old name: o-aminoazotoluene.

this group, hepatomas 123 and 138, grew only upon subcutaneous transplantation. The last two hepatomas (120 and 121) failed to grow in any form upon either intraperitoneal or subcutaneous transplantation. Thus, from these 16 hepatomas, 2 strains, and 2 sublines from 1 of these strains, were established as true ascites tumors, and these have now been carried by transfer of the tumor ascites from 60 through more than 90 generations.

TABLE 1.—*Conversion of mouse hepatomas into ascites form*

Group	No.	Growth of tumors upon intraperitoneal transplantation		Approximate survival time (*days*)	Number of transplant generations
		In ascites form	In solid form		
A	134	+*	+	14†	90
	129	+*	+	17†	60
	129(P)	+	+	17†	90
	129(F)	+	+		80
B	122	−‡	+	120	2
	128	−‡	+	90	1
	136	−‡	+	120	1
	140	−‡	+	60	2
	147	−‡	+	30–50	4
C	101	−	+	90	2
	108	−	+	120	1
	118	−	+	120	1
	119	−	+	150	1
	123	−	−§	180	1
	130	−	+	120	1
	138	−	−§	80	2
D	120	−	−	—	1
	121	−	−	—	1

*Immediate conversion into ascites form.
†Fifty percent survival.
‡A few islands or clumps of tumor cells appeared in peritoneal fluid.
§Tumors inoculated subcutaneously, grew as solid tumors.

Mouse ascites hepatoma 134.—Hepatoma 134, obtained from Dr. H. B. Andervont, originated as an induced liver tumor in a C3H female mouse following administration of carbon tetrachloride (*9*) and had been carried subcutaneously for three generations. For the initial intraperitoneal transplantation, a 42-day-old solid tumor was used as described above. Two days after inoculation into five mice, the volume of ascitic fluid in each mouse had increased slightly and appeared as a very thick, white suspension of single tumor cells (fig. 9) and many small tumor-cell aggregates or "islands" (figs. 13 to 16). Some islands were round in shape while others consisted of several cells linked together to form chains (figs. 14 and 15). While the size of such islands was small when observed a few days after transplantation, much larger aggregates were formed during the next several days of development. Mitotic figures, while not abundant in the early generations, could nevertheless be detected on Giemsa-stained smears in both free cells and cell islands. Thereafter,

the ascitic fluid increased in amount daily, swelling of the abdomen became apparent, and the number of tumor cells and tumor islands increased. Eight days after inoculation, the original white, milky ascites had become hemorrhagic, and this condition persisted to the end. Microscopically, at 8 days the smallest unit consisted of 2 cells, whereas the larger islands contained 50 to 60 or more cells. The majority of tumor cells measured about 20 to 40 μ in diameter, but often giant cells measuring 50 to 70 μ in diameter with 1, 2, or more nuclei were observed.

Upon establishment in the peritoneal cavity, serial intraperitoneal transplantation with the tumor ascites was initiated; this tumor is now in the 90th transplant generation.

The amount of tumor ascites ordinarily used for each inoculation was about 0.05 cc. The mice died with 3 to 5 cc. of tumor ascites, exhibiting tumor invasion to various tissues in the peritoneal cavity, *i.e.*, portal area, omentum, retroperitoneum, and mesentery. In addition, many small, white nodules were visible on the peritoneal surface. A characteristic of this ascites hepatoma was its ability to invade the diaphragm; tumor cells which had penetrated through the diaphragm and into the pleural cavity were often observed proliferating in the pleural fluid.

Mouse ascites hepatoma 129.—Hepatoma 129, obtained from Dr. A. J. Dalton, originated in Dr. Andervont's laboratory as an induced liver tumor in a C3H male mouse after administration of carbon tetrachloride (*9*) and had been carried subcutaneously through 25 passages. This tumor had been converted into the ascites form by Dalton and Felix, who also observed the formation of hepatoma islands.[7]

In the present experiments a 17-day-old solid tumor was used for intraperitoneal transplantation into each of 5 mice. A few days after transplantation, the ascitic fluid appeared as a thin, slightly hemorrhagic suspension containing many floating cell islands (figs. 11 and 12) visible to the naked eye. The majority of these islands were considerably larger than those of hepatoma 134, even after the latter had attained their maximum size.

Many islands contained more than 100 cells. Cell size was similar to that of hepatoma 134 and the ascites similarly contained large (giant) cells. Following intraperitoneal transplantation with the tumor ascites, mice died with the development of an extremely hemorrhagic ascites accompanied by tumor invasion to various sites in the peritoneal cavity, similar to hepatoma 134, *i.e.*, portal area, peritoneal surface, omentum, retroperitoneum, and mesentery. In addition, invasion of the diaphragm was a marked and consistent feature. The intrapleural proliferation of tumor cells which had penetrated the diaphragm, as well as a few cases of metastases to the liver and kidney, were also observed.

Mouse ascites hepatoma 129(P).—In the course of the serial intraperitoneal transplantation of the island ascites hepatoma 129, one female C3H mouse out of a group of five, in the third transplant generation, exhibited an unusually long survival time. Thus, while the other 4 mice

[7] Personal communication.

died within a typical period of 18 to 28 days, this mouse survived for 50 days. At autopsy, just after death, a solid tumor (2 × 2 cm.) was observed on the abdominal wall, together with heavy tumor invasion to the omentum, mesentery, retroperitoneum, portal area, and diaphragm. In addition, however, a quantity of fluid was present in both the peritoneal and pleural cavities. The fluid of the pleural cavity was actually a very thick, milky white suspension not only of many islands but also of free tumor cells. With this pleural fluid, intraperitoneal transplantation into new mice was begun and, after several days, a free-cell ascites hepatoma developed (fig. 10). Although a few islands could still be found in the ascites, most of these were small complexes as shown in figures 17 to 20 and in no way compared with the enormous islands which marked the ascites of hepatoma 129 from which it had originated. The nature of the tumor ascites had also changed, for, whereas the fluid of the original island ascites (129) was thin and hemorrhagic, the ascites of the new, free-cell subline, was thick and milky white, even in the end stage of tumor growth. This subline, 129(P), has been carried intraperitoneally through more than 90 generations and the characteristics described have thus far remained unchanged.

At the time this free-cell hepatoma, 129(P), developed, it was observed that the majority of these cells reacted in a different fashion when stained with Giemsa. A thin, pink-stained layer appeared fringing the blue-stained cytoplasm. Cytochemical tests for glycogen and other polysaccharides (with periodic acid-Schiff reagent) and for lipide (with Sudan black) were negative in this particular zone. In addition a number of other dyes, *i.e.*, neutral red, acetic orcein, etc., gave no observable indication of any change in this peripheral zone. The meaning of this observation is not apparent, although it may be inferred that these changes in the peripheral zone of the cytoplasm, appearing concomitantly with the transformation into a free-cell condition, indicate certain changes in the cell surface that took place at this time.

Mouse ascites hepatoma 129(F).—As described above, mouse ascites hepatoma 129 was an ascites tumor composed of very large islands. In the early experiments, a few free tumor cells were sometimes observed in the ascites but, except for the unexpected change in one mouse which led to the development of hepatoma 129(P) in the third generation, the original tumor line remained in an island form for about 30 subsequent generations (a time interval of about 10 months).

Following this, difficulties were experienced in obtaining an adequate supply of pure strain C3H mice; consequently, for several generations noninbred C3H mice had to be used for inoculation. Intraperitoneal transplantation during this period did not result in 100 percent tumor "takes" as had been the case with the use of inbred mice. During this period, between the 35th and the 40th transfer generations, a change occurred in the tumor islands in all the mice used for inoculation. An increase in free tumor cells was observed with a concomitant decrease in the number of tumor islands to a point where they disappeared almost

entirely from the ascites. It should be noted, however, that this change to a free-cell condition, once initiated, progressed gradually through several generations, during which time the tumor ascites was a mixture of free cells and tumor islands. When the tumor ascites finally emerged as a free-cell tumor, it appeared to be almost identical with the first subline, hepatoma 129(P).

Recently, we attempted again to convert solid hepatoma 129 into the ascites form. This conversion resulted in a thin, hemorrhagic exudate containing tumor islands, the appearance of which was similar to that obtained in the first conversion of this tumor. Likewise, this tumor line could be carried by serial intraperitoneal transplantation.

At the seventh generation, however, the tumor islands of several, but not all, of the inoculated mice again changed rather abruptly into a mixture of islands and many free cells.

Nonconvertible hepatomas.—Of the mouse hepatomas which could not be established as ascites tumors, it appeared that some might have been capable of conversion had the tumor ascites been passed through several further generations. For example, hepatoma 147 (*9*), inoculated intraperitoneally, required a longer time to "take" (30 to 50 days), but a number of tumor islands similar to those of hepatoma 129 were always observed floating in the ascites. This tumor ascites was transferred through four generations and then discontinued. In mouse hepatomas 140 and 122, hemorrhagic ascites and some tumor islands could be found 2 and 4 months, respectively, after intraperitoneal inoculation. However, these tumors, as well as the other hepatomas which failed to convert, might not have been carried a sufficient length of time to provide evidence of their transformation into ascites tumors.

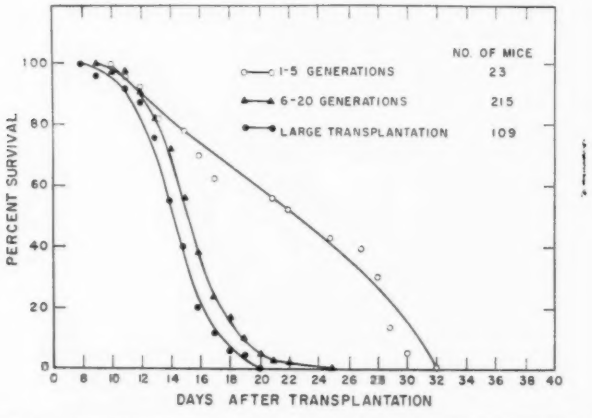
The question of factors concerned in determining whether the inoculated tumor cells can proliferate in the ascitic fluid has been discussed by Klein, who has indicated that transformation into the ascites form occurred only with rapidly growing tumors (*2*). The present data appear to agree with this conclusion. Thus, only hepatomas 134 and 129, which were relatively fast-growing tumors, could be established in the ascites form. Relatively slow-growing tumors, such as hepatoma 147 and others, failed to convert within the limited terms of the experiments. However, the work of Klein on the "gradual transformation" of tumors indicates that some of these might have converted had the intraperitoneal passages been continued (*4*).

Survival time of mice bearing ascites hepatomas.—Changes in rapidity of growth of newly established tumors upon successive transplantation have been observed by numerous workers. Thus, for example, Andervont and Dunn have noted the ability of mouse hepatomas to grow faster after the first few transplant generations (*10*). In the case of the Yoshida sarcoma, however, the percentage of tumor "takes" even in the earliest generations remained high, and successive transfers did not appear to increase the growth speed of the tumor. Yoshida has interpreted these results to indicate that, from the time of its inception, the malignancy

of this tumor, as measured by transplantability, has remained essentially unchanged.

In studying the growth speed and transplantability of a tumor, consideration must also be given to both the tumor and the strain of animal used. If the animals are not of a pure, inbred strain, then susceptibility of such animals to a tumor should be considered before the growth capacity of the tumor cells is determined. It is also clear that the use of pure-strain animals does not always ensure a high percentage of transplantability. Thus, in the transplantation of mouse hepatomas, the inability of grafts from primary tumors to "take," even when inoculated into pure-strain animals, has been observed (*10*).

The conversion of solid tumors into a fluid form obviously involves an environmental change of some consequence, and we were interested in establishing two points: first, whether the "immediate" conversion into ascites form would preclude the possibility of further changes in the

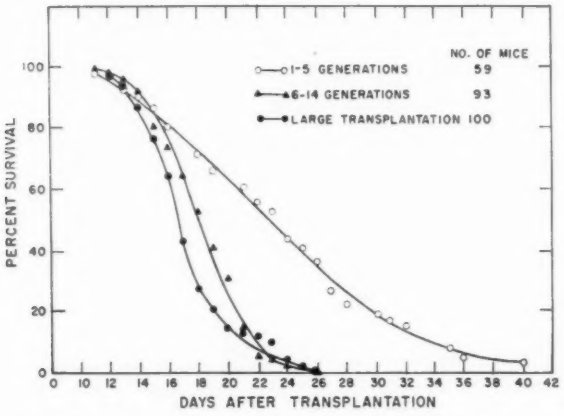


TEXT-FIGURE 1.—Survival curve of mice intraperitoneally transplanted with ascites hepatoma 134.

growth potential of the tumor as measured by transplantability; second, the percentage of ascites "takes," as compared with the takes of solid hepatomas, that would be obtained with the use of pure-strain animals in both cases.

The survival curves of three ascites hepatomas, 134, 129, and 129(P), are shown in text-figures 1 to 3. To illustrate the results more clearly, we first examined the survival data in each generation to determine at what point the obvious changes in survival time occurred. Then the survival curves were grouped together for simplicity at points where changes in survival time were most apparent. These data fall into three groups: (*a*) one through five or seven generations, (*b*) several subsequent generations, and (*c*) a group containing a large number of mice which were transplanted at one time, several generations after each tumor line had become definitely established.

In each ascites tumor, continued intraperitoneal passage resulted in a decrease in survival time. The survival time of 50 percent of the animals is at present about 14 days in hepatoma 134 and about 17 days in both hepatomas 129 and 129(P). Despite such alterations in survival times, 100 percent "takes" were obtained almost immediately in each of the tumors used. At present, all three ascites hepatomas "take" 100 percent when inoculated intraperitoneally into C3H/HeN mice. One may consider the decrease in survival time as a sign of the increase in malignancy of the tumor cells or as the result of the selection, over a period of time, of those cells best capable of growth in the ascites form. The possibility also exists that the malignancy of the tumor cells is essentially unchanged but that such cells, placed in a new environment, require a period of time for further adaptation.



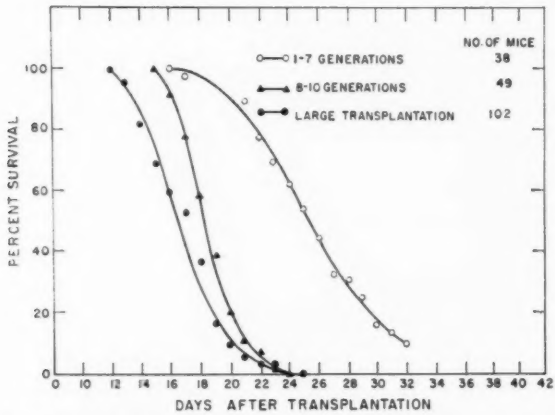
TEXT-FIGURE 2.—Survival curve of mice intraperitoneally transplanted with ascites hepatoma 129.

Histologic problems.—We were interested in studying the histologic sections of these mouse hepatomas to determine whether any morphologic differences were discernible between the tumors which were successfully converted into the ascites form and those which failed to grow in a fluid condition.

In reviewing the hepatomas used in our experiments, the histologic patterns of the tumors appeared as "well-differentiated" hepatomas as shown in figure 1. Hepatoma 123 failed to grow in the ascites form. Figures 3 and 4 illustrate areas of hepatoma 129, a tumor which was successfully and immediately converted into an ascites tumor. Although differences are apparent between the pictured areas of these 2 tumors, a careful study of many sections of hepatoma 129 and all the nonconvertible hepatomas revealed no consistent differences by which these could be distinguished from each other. All such tumors reproduced the architecture of the liver parenchyma and were recognized as hepatomas.

Nevertheless, some morphologic differences were suggested when a number of sections from each tumor were compared. Thus, for example, in the unconverted tumors the sinusoidal space between the parenchymal tumor cells appeared much wider and more regular. Also, in hepatoma 134 (fig. 2) the histologic pattern appeared to be more anaplastic, *i.e.*, the cordlike arrangement of tumor cells was smaller and more irregular than that of hepatoma 123 and the liver architecture was not so clearly reproduced.

Although in general the microscopic examination of these parent tumors failed to reveal any consistent differences between the successfully converted tumors and those which were unsuccessful, some interesting changes were observed, particularly in the case of hepatoma 129(P). As described above, this hepatoma was separated as a subline from a mouse bearing hepatoma 129. This mouse survived an unusually long



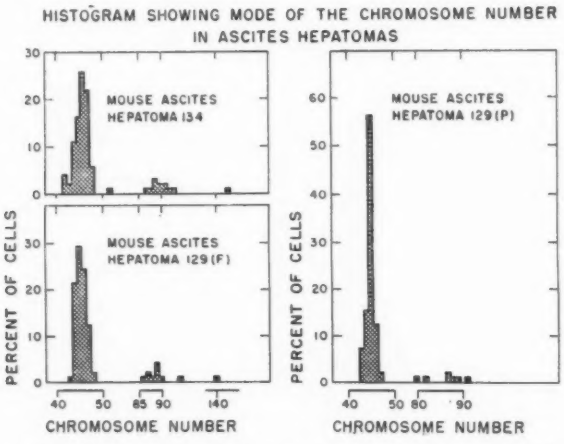
TEXT-FIGURE 3.—Survival curve of mice intraperitoneally transplanted with ascites hepatoma 129(P).

period of time during which the islands of the ascites were largely replaced by a population of single cells. When the animal died, the solid-tumor tissue which had invaded the diaphragm, peritoneal surface, omentum, mesentery, and portal areas was examined. In addition to those areas, which resembled in structure the original tumor (129), a proliferation of fusiform cells was also observed. Figures 5, 7, and 8 show several portions of ascites hepatoma 129(P) reconverted into solid form subcutaneously; in figure 8, the appearance of a sarcoma-like architecture is particularly evident. In figure 6, the same tumor is shown invading the diaphragm. This change in histologic pattern appeared to be correlated with the alteration from island to free-cell growth. Similar changes were also observed in the histologic pattern of ascites hepatoma 129(F), which was separated as a second subline from hepatoma 129 after the 40th generation. Here again, a histologic section of the ascites tumor reconverted into solid form reveals areas resembling well-differ-

entiated hepatoma, as well as relatively more anaplastic areas and a sarcoma-like structure composed of fusiform cells.

The mode of the chromosome number.—Chromosome studies were carried out using a hypotonic method. About 1 cc. each of 5-day-old ascites from hepatomas 134, 129(F), and 129(P) were mixed with twice the volume of 0.2 to 0.5 percent saline. After 5 to 15 minutes, 1 percent aceto-orcein was added. The chromosomes were well spread and their number and shape readily determined (figs. 21 to 26).

For a systematic study of chromosome number, the ascites used were those of the 50th generation in hepatoma 134, the 40th in hepatoma 129(P), and the 48th in hepatoma 129(F). In each tumor 100 metaphase plates were examined. Results of the chromosome counts are shown in text-figure 4. In all three strains the range of chromosome numbers was essentially similar, and about 10 percent of the mitotic plates possessed a near-tetraploid number of chromosomes. Special attention was paid to the appearance of the shape of chromosomes in the nuclear plates examined. In both hepatoma 129(F) and hepatoma 129(P) all of the chromosomes were in the shape of long or short rods, while in hepatoma 134 a few nuclear plates containing one or two V-shaped chromosomes were also observed occasionally.



TEXT-FIGURE 4.—Histogram showing mode of the chromosome number in ascites hepatomas.

Discussion

In studies on the conversion of rat hepatomas into the ascites form, it was observed that not all of the primary liver tumors could be transformed into the ascites form (*11,3*). Some grew only as solid masses in the peritoneal cavity and failed to develop into a true ascites tumor. During the limited terms of the present experiments, similar results were obtained. In several cases the formation of tumor islands in the ascites was observed but these later disappeared and a true ascites tumor failed to develop.

However, in view of Klein's recent findings (*4*) a small number of tumor islands might have produced an ascites tumor had the peritoneal exudate been carried through sufficiently long-continued serial passage.

In the successfully converted tumors, it appeared that the rapidity of growth of the parent tumor influenced the development of ascites tumors, for both hepatoma 129 and hepatoma 134 were relatively fast-growing tumors. Anaplasia, however, did not seem to be a factor in ascites-tumor formation. Indeed, hepatoma 129 and, to a somewhat lesser degree, hepatoma 134 were well-organized tumors but, despite this fact, both were converted immediately into the ascites form.

Most ascites tumors grow essentially in a free-cell condition, but cells of the ascites hepatomas of the rat and mouse clearly maintain their epithelial nature to a larger extent. In view of this, the free-cell ascites-hepatoma sublines which arose spontaneously in the ascites of hepatoma 129 were studied. Two possibilities as to their origin are immediately apparent: 1) the free-cell population was present but masked within the islands of hepatoma 129 or 2) the cells of the tumor islands themselves underwent some type of alteration which led to the free-cell condition.

A comparison of the histologic sections of hepatoma 129 with those of the free-cell sublines reconverted into solid form suggests only that the free-cell tumors appear to be less differentiated than the original tumor. Although occasional sarcoma-like areas were detected, most areas in the section studied indicated that the tumor was still essentially epithelial in nature.

Summary

1) Attempts to convert 16 mouse hepatomas into the ascites form have been described. Two of these 16 gave rise immediately to island ascites tumors, while the remaining hepatomas either failed to grow or produced solid-tumor tissue following intraperitoneal inoculation.

2) From the island ascites tumor of one such hepatoma, two free-cell sublines were derived.

3) The survival times of both ascites hepatomas and of a single subline decreased after serial transplantation but the percentage of "takes" was 100 throughout the course of transplantation.

4) Histologic inspection failed to reveal any correlation between anaplasia of the hepatomas and the ability to form ascites tumors. The two ascites-hepatoma sublines, when reconverted into solid form, appeared to be less differentiated than the island hepatoma from which they had originated. In addition, some areas possessed a sarcoma-like appearance, but in general the tumors retained epithelial characteristics.

5) The modes of the chromosome numbers in one ascites hepatoma and two sublines was essentially similar, and about 10 percent of the mitotic plates possessed a near-tetraploid number of chromosomes.

6) Growth speed of the hepatomas appeared to be an important factor in their ability to form ascites tumors.

7) The role of anaplasia and growth speed of hepatomas in the formation of ascites tumors is discussed. Possibilities as to the nature of the change from an island ascites tumor to a free-cell condition are suggested.

References

(*1*) KLEIN, G.: Development of a spectrum of ascites tumors. Exper. Cell Res. 2: 291–294, 1951.

(*2*) ———: Conversion of solid into ascites tumours. Nature, London, 171: 398–399, 1953.

(*3*) YOSHIDA, T.: Contributions of the ascites hepatoma to the concept of malignancy of cancer. Ann. New York Acad. Sc. 63: 852–881, 1956.

(*4*) KLEIN, E.: Gradual transformation of solid into ascites tumors. Evidence favoring the mutation-selection theory. Exper. Cell Res. 8: 188–212, 1955.

(*5*) ———: Immediate transformation of solid into ascites tumors. Studies on a mammary carcinoma of an inbred mouse strain. Exper. Cell Res. 8: 213–225, 1955.

(*6*) SATO, H., and ARUJI, T.: Studies on the ascites hepatoma. Gann 43: 254–257, 1952.

(*7*) ARUJI, T.: Experimental transformation of DAB-hepatoma of rats into the ascites form. (In Japanese) Gann 44: 130–132, 1953.

(*8*) SATO, H.: Intraperitoneal transplantation of the Yoshida ascites sarcoma and the ascites hepatoma to various American strains of rats. J. Nat. Cancer Inst. 15: 1367–1378, 1955.

(*9*) ANDERVONT, H. B., and DUNN, T. B.: Transplantation of hepatomas in mice. J. Nat. Cancer Inst. (Suppl.) 15: 1513–1524, 1955.

(*10*) ———: Transplantation of spontaneous and induced hepatomas in inbred mice. J. Nat. Cancer Inst. 13: 455–503, 1952.

(*11*) YOSHIDA, T., SATO, H., and ARUJI, T.: Origin of the Yoshida sarcoma. I. Experimental production of "ascites hepatoma" in the rat. Proc. Japan Acad. 27: 485–492, 1951.

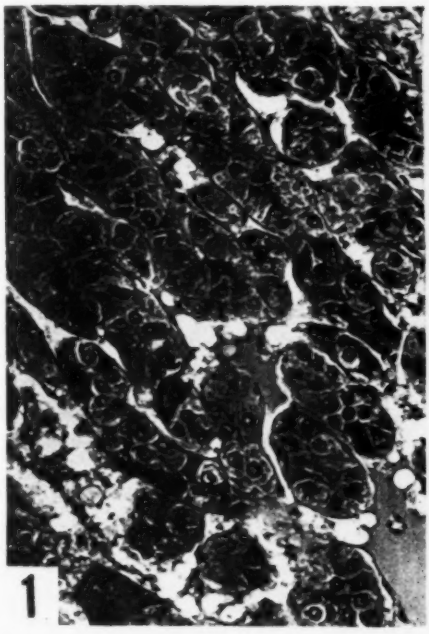
PLATE 1

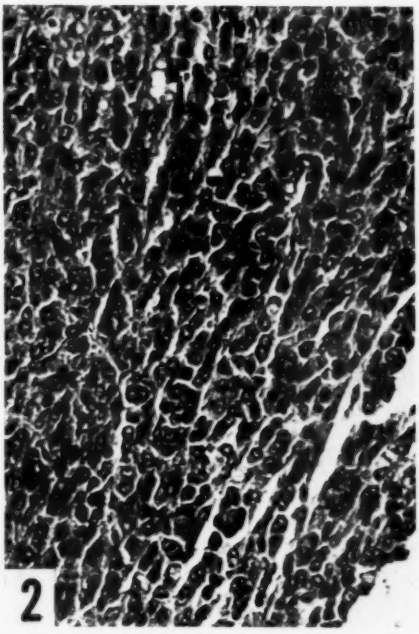
FIGURE 1.—Hepatoma 123. Hepatoma which failed to convert into ascites form. Arrangement of cells and cytologic characteristics closely resemble normal liver. Hematoxylin and eosin. × 300

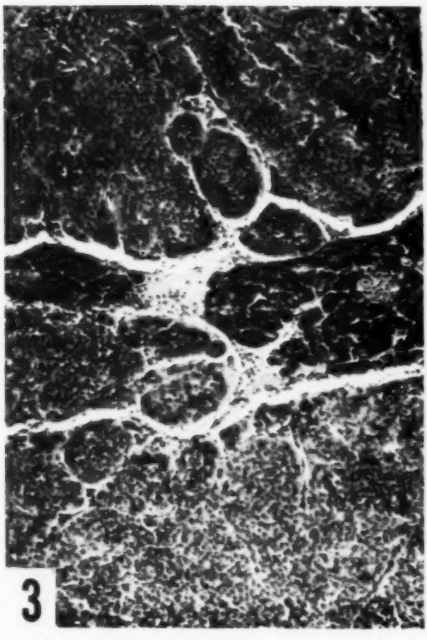
FIGURE 2.—Hepatoma 134. Converted into ascites form in the first intraperitoneal transplantation. Individual cells resemble those of normal liver, but cordlike arrangement of cells is less evident than in hepatoma 123. Hematoxylin and eosin. × 100

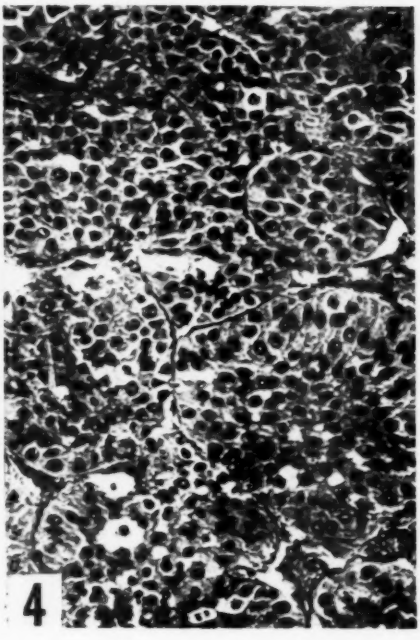
FIGURE 3.—Hepatoma 129. Shown invading the tissue surrounding pancreas. Cells of this tumor resemble liver cells but are arranged in nests rather than cords. Hematoxylin and eosin. × 100

FIGURE 4.—Hepatoma 129, original tumor. Also converted into ascites form in the first intraperitoneal transplantation. Individual cells have characteristics of liver cells, but the arrangement in nodules, separated by fine connective-tissue bands, does not resemble the normal liver-cell arrangement. Hematoxylin and eosin. × 300









38636—56——2

PLATE 2

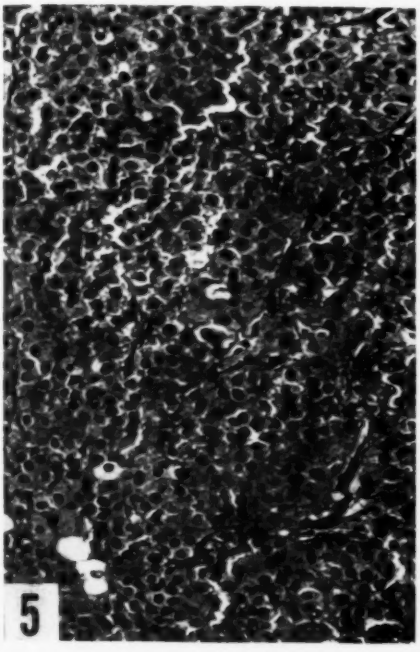
Ascites hepatoma 129(P) growing in solid form

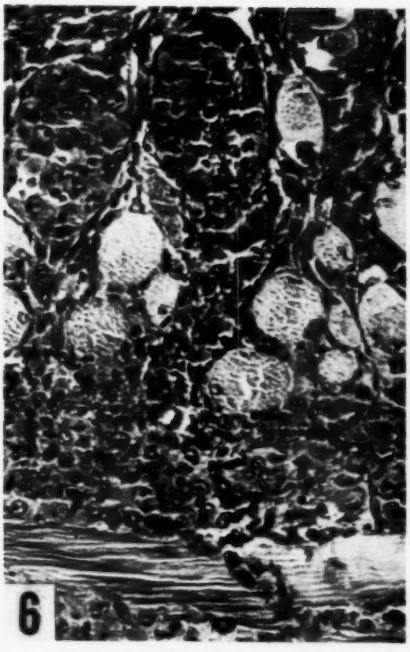
FIGURE 5.—Subcutaneous. An area similar to original tumor (hepatoma 129), but cells appear smaller and more deeply stained. Hematoxylin and eosin. × 300

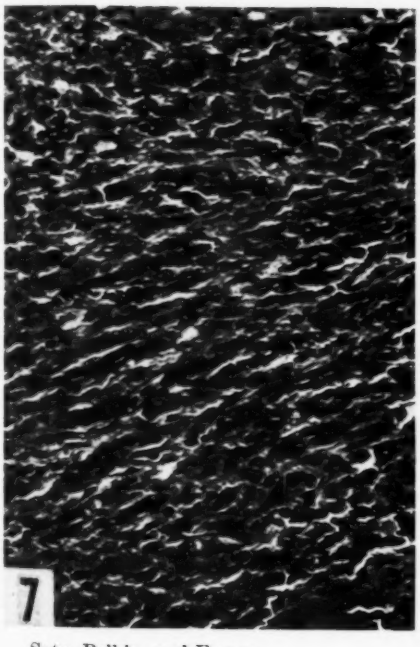
FIGURE 6.—Invasion into diaphragm. Cells are rounded, invading muscle, and resemble anaplastic carcinoma. Hematoxylin and eosin. × 300

FIGURE 7.—Subcutaneous. Cells showing tendency toward fusiform structure. Hematoxylin and eosin. × 300

FIGURE 8.—Subcutaneous. Cells more obviously fusiform and resemble sarcoma. Hematoxylin and eosin. × 300


5


6


7

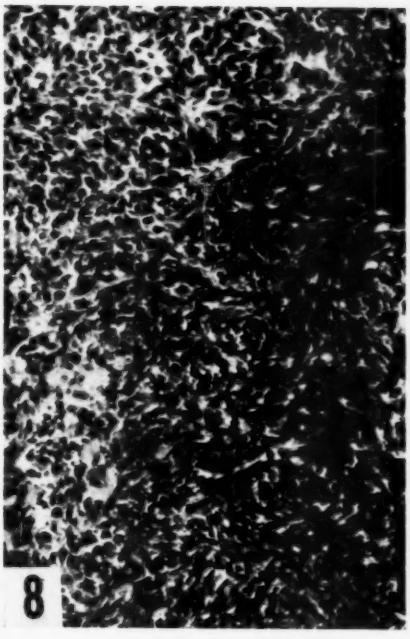

8

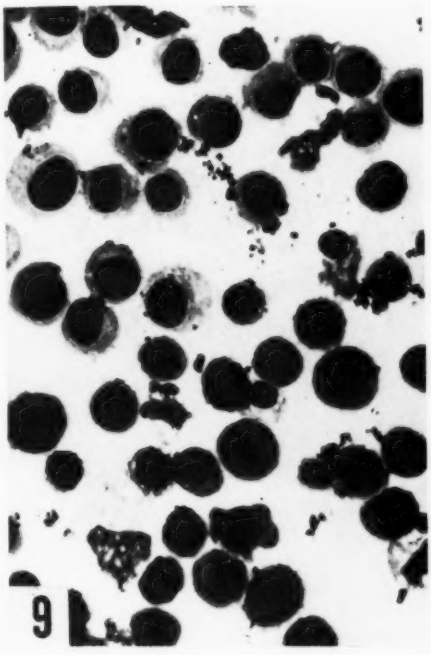
PLATE 3

FIGURE 9.—Mouse ascites hepatoma 134. Smear from a thick, milky ascites showing area of single tumor cells. Giemsa. × 500

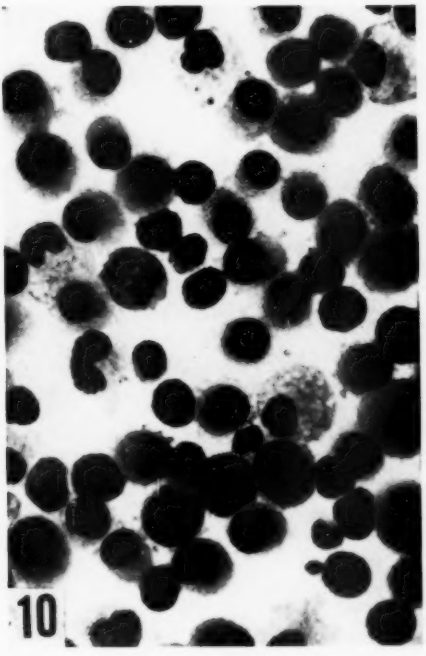
FIGURE 10.—Mouse ascites hepatoma 129(P). Free-cell subline derived from islands of ascites hepatoma 129. Giemsa. × 500

FIGURE 11.—Mouse ascites hepatoma 129. Island ascites hepatoma. Aceto-orcein. × 60

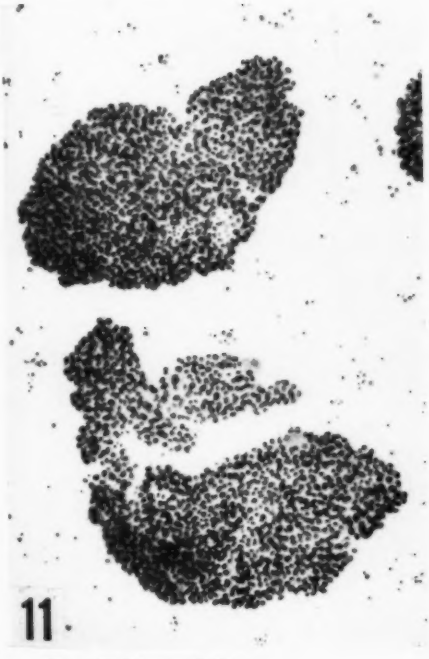
FIGURE 12.—Mouse ascites hepatoma 129. An island composed of about 100 tumor cells. Aceto-orcein. × 200



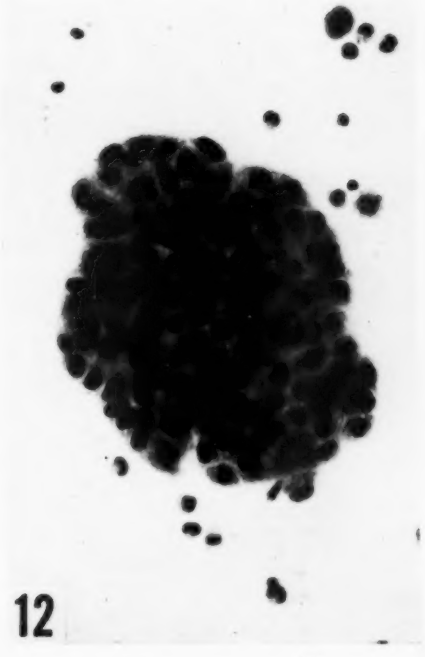
9



10



11



12

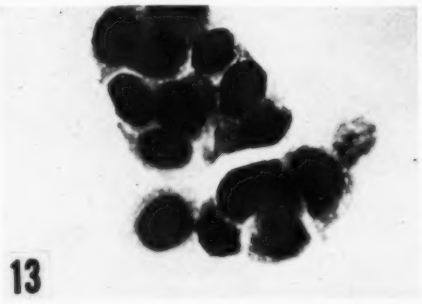
PLATE 4

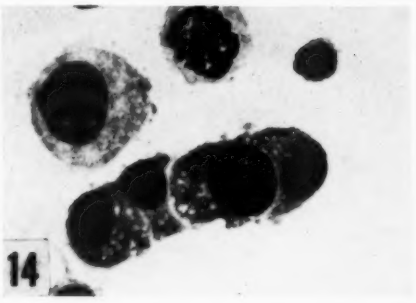
Mouse ascites hepatoma 134

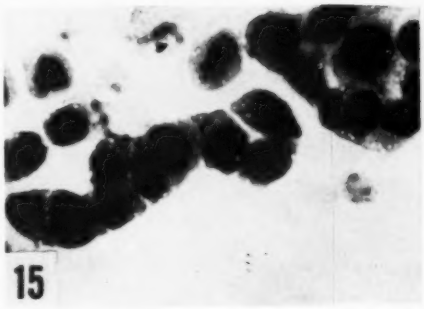
FIGURES 13 TO 16.—Various types of small aggregates of tumor cells observed 2 days after intraperitoneal inoculation of material from solid hepatoma 134. Giemsa. × 800

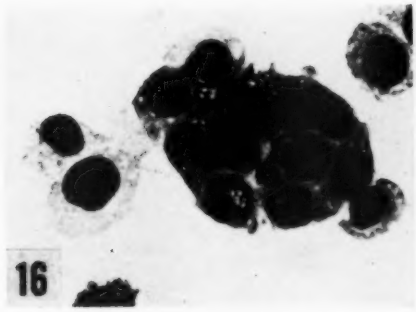
Mouse ascites hepatoma 129(P)

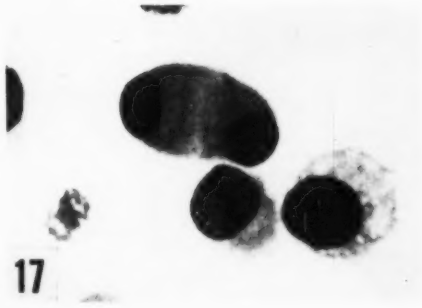
FIGURES 17 TO 20.—Various types of small aggregates of tumor cells derived from intraperitoneal inoculation of solid hepatoma 129. Compare with size of islands in figures 11 and 12. Giemsa. × 800


13

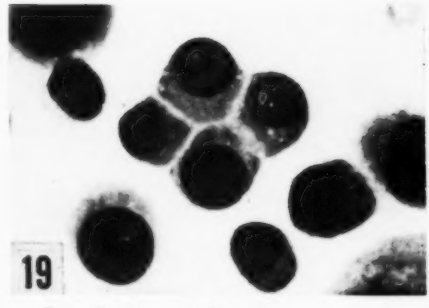

14


15


16


17

18


19

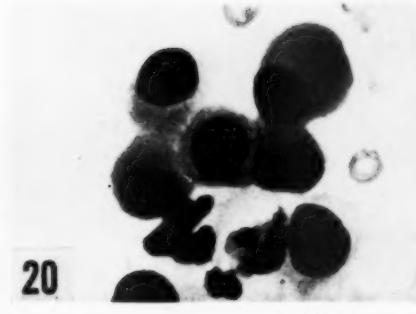

20

PLATE 5

Chromosome plates in ascites hepatoma 134

FIGURE 21.—Nuclear plate with near-diploid number of chromosomes. Chromosome number, about 43. Aceto-orcein squash. × 1,500

FIGURE 22.—Nuclear plate with near-diploid number of chromosomes. Chromosome number, about 46. Aceto-orcein squash. × 1,500

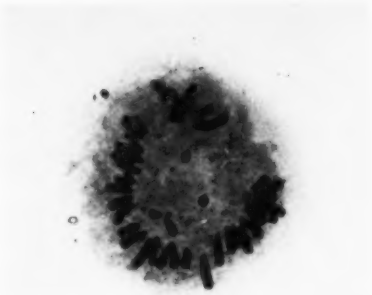
FIGURE 23.—Nuclear plate with near-tetraploid number of chromosomes. Chromosome number, about 105. Aceto-orcein squash. × 1,200

Chromosome plates in ascites hepatoma 129(P)

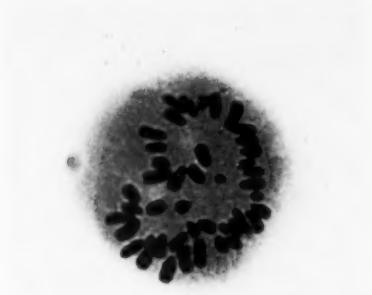
FIGURE 24.—Nuclear plate with near-diploid number of chromosomes. Chromosome number, about 45. Aceto-orcein squash. × 1,500

FIGURE 25.—Nuclear plate with near-diploid number of chromosomes. Chromosome number, about 45. Aceto-orcein squash. × 1,500

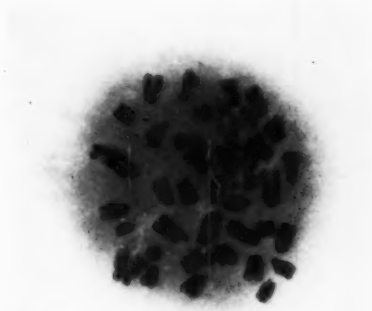
FIGURE 26.—Nuclear plate with near-tetraploid number of chromosomes. Chromosome number, about 91. Aceto-orcein squash. × 1,500



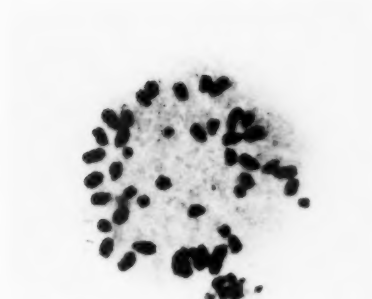
21



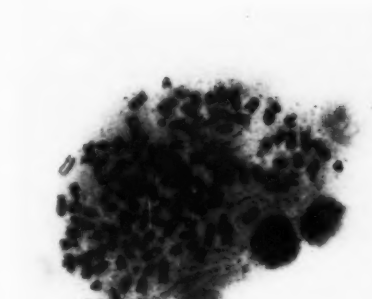
24



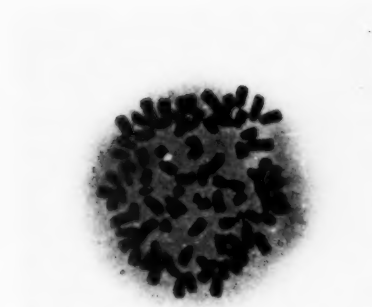
22



25



23



26

386036—56——3

The Role of Graft and Host Vessels in the Vascularization of Grafts of Normal and Neoplastic Tissue [1]

RUTH M. MERWIN *and* GLENN H. ALGIRE, *National Cancer Institute,*[2] *Bethesda, Maryland*

Observations on the vascularization of isografts [3] of normal and neoplastic tissues are reported in this paper. Studies were made primarily on grafts of small fragments of thyroid or mammary adenocarcinoma tissue placed in transparent chambers in the skin of mice. Three problems in particular were considered.

First, observations were made on the vessels that survived in grafts of normal tissues. Although most authors have recognized that these vessels survive, almost all have assumed that only the vessels of the hosts are active in establishing connections between graft and host vessels. However, in a report preliminary to the present paper (*1*) and in a paper by Williams (*2*) it was pointed out that the vessels in the grafts may also be active.

Second, since new findings were made concerning the surviving vessels in grafts of normal tissue, it seemed pertinent to make further observations on the vessels in grafts of tumor tissue. Previous evidence indicated that the vessels in tumor tissue do not even survive. It was found here, however, that some vessels may survive in some tumor grafts, although these surviving vessels do not seem to form new vessels.

Third, the effect of grafts of normal and neoplastic tissue on adjacent host vessels was studied. The main finding confirms previous reports in the literature (*3,4*) that grafts of tumor tissue stimulate the proliferation of host vessels more than do grafts of normal tissue.

Materials and Methods

A transparent-chamber method (*5*) and a modification of it (*6*) were used to observe the vascularization of grafts in living mice. In the original method the skin is held up continuously in a double-layered flap along the middorsal line. An incision is made in one layer of skin which exposes a considerable area of the inside surface of the other layer. A graft is embedded in the exposed layer and a glass disc, inserted through the inci-

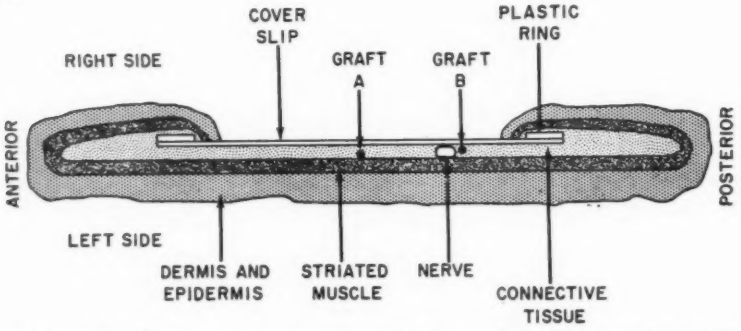
[1] Received for publication February 2, 1956.

[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

[3] Isografts are grafts between animals of the same inbred strain. Homografts are grafts between animals of the same species but not the same inbred strain.

386036—56——4

sion, covers the graft and the entire exposed area (see text-fig. 1). In the modified method the skin is allowed to return to its normal position so that the incision and glass window are on one side of the mouse and the graft is on the other side about 7 mm. from the midline. In order to observe the graft through the window, the skin is temporarily held up in a dorsal flap. The time and manner of vascularization of grafts was the same whichever method was used. Although the second method was easier, photographic records were difficult to obtain and microscopic observations were limited to the use of a stereoscopic microscope at a magnification of 90.



TEXT-FIGURE 1.—Diagram of a section through a transparent chamber in the dorsal skin flap of a mouse to show the relationship of the skin to the chamber and to show the position of grafts. The nerves, which in this area of the skin run ventrodorsally in the subpannicular layer of connective tissue, are seen in cross section. Graft A is embedded in the connective tissue close to the striated muscle layer, the panniculus carnosus. Graft B is embedded close to a nerve and as far as possible from the muscle layer.

Grafts were embedded in the subpannicular layer of connective tissue, which is avascular except for the vessels that accompany nerves. In previous work in this laboratory each graft was placed like graft A, text-figure 1, so that vascular connections were established between the vessels of the graft and those of the host muscle layer. The area in which the connections formed could be seen only by focusing through the graft. To avoid having to focus through the graft, most of the implants in the present experiments were placed like graft B, so that vascular connections were established between the vessels of the graft and those of a host nerve. The placing of grafts in this manner did not seem to injure the host vessels.

The diameter of grafts in the plane of the windows ranged from 0.5 to 1.5 mm., but the thickness of the grafts was considerably less than this. Such small grafts survived whether or not they became vascularized.

A graft was considered vascularized when blood from the host was found in vessels within the graft, regardless of whether the blood was circulating or whether the vessels were provided by the host or by the graft. The vessel sprouts referred to in this paper are canalized. The solid threads of endothelium, which precede the canalized sprouts in the formation of new vessels, could not be seen with the methods used.

Observations were made on 25 grafts of a mammary adenocarcinoma, C3HBA (*7*), placed adjacent to nerves and on 35 similarly placed grafts of thyroid tissue from either adult mice or young mice less than 3 days old. For comparative purposes observations were made not only on other grafts placed at different sites or placed in previously injured areas but also on sprouts growing from vessels in cut nerves.

Mice of the C3H/HeN strain (*8*) were used. The hosts in each of the experimental groups ranged in age from 2 to 5 months. The age of the host seemed to have no effect on the vascularization of grafts.

In order to maintain the temperature of the skin in the area of the transparent chamber, where the skin was pulled up away from the body and where the hair had been removed, the mice were placed in an incubator at a temperature of 30° C. The mice with modified chambers were also placed in an incubator.

Observations and Results

Grafts of Thyroid Tissue

Most grafts of thyroid tissue were vascularized if the grafts were not over 50 μ from the vessels of the host nerves. Almost none were vascularized, however, if the grafts were more than 50 μ from the host vessels. Twenty-five grafts of thyroid tissue from young mice 1 to 3 days old were placed approximately 50 μ or less from host vessels. Within 1, 2, 3, and 4 days after grafting 3, 39, 58, and 65 percent, respectively, were vascularized. Of 10 grafts of adult thyroid tissue only 2, that is 20 percent, were vascularized within 4 days. These 2 were vascularized on the 3rd day. Vascular connections between the vessels of the grafts and those of the hosts were established by many short vessels. The origin of these vessels was not evident by direct observation because all the connections were completed when the vessels were first seen and, in this immediate area, no sprouts grew from either the graft or the host vessels.

The vessels of the host nerves in the vicinity of the grafts produced no sprouts, but some vessels became slightly dilated and convoluted. The vessels in grafts, on the other hand, produced many sprouts, although none grew on the side of the grafts where the connecting vessels formed. Graft vessels and their sprouts were visible even before the grafts were vascularized because they contained donor blood. This donor blood seemed to enter the sprouts as soon as they became canalized. During the first day after grafting, no sprouts formed. During the second day, a few sprouts grew from the vessels in some grafts of young thyroid tissue. Some of these sprouts were 140 μ long. Not until the third day did any sprouts grow from vessels in grafts of adult tissue. After grafts were vascularized the sprouts that had already formed continued to grow but no new ones appeared.

Almost none of these sprouts joined the vessels of the adjacent host nerves because all the sprouts grew parallel to the nerves. No sprouts emerged from the sides of the grafts either toward or away from the nerves

(see fig. 1). The cells and fibers of the host connective tissue around the nerves were oriented parallel to the nerves, as were the fibrils of fibrin that formed in the area around grafts. If two grafts were placed on the same side of a nerve and within a few millimeters of each other, sprouts arising from the two grafts and growing parallel to the nerve anastomosed with each other. The pattern of outgrowth of sprouts was different for grafts placed at other sites. For instance, grafts placed far from nerves, although similarly embedded in the same layer of connective tissue, produced only a few short sprouts. These sprouts grew in a circular path close to the edge of the grafts.

Although nonvascularized grafts of thyroid tissue that were adjacent to the vessels in nerves rarely affected the host vessels, similar grafts that were close to the muscle layer, like graft A in text-figure 1, caused rich networks of host vessels to develop after a few weeks. These networks in the host muscle layer could be seen by focusing through the grafts. Sprouts never grew toward the grafts from such networks. The cells and fibers of the intervening connective tissue were oriented in a plane parallel to the muscle layer, and apparently new sprouts could not grow at right angles to this plane.

A few observations made on grafts of other glandular tissues, such as harderian gland and salivary gland, indicated that the vascular changes in both the grafts and hosts were similar to those for grafts of thyroid tissue.

Grafts of Tumor Tissue

Grafts of tumor tissue placed close to the vessels of nerves caused a rich network of new vessels to form (see fig. 2). This is in striking contrast with the reaction of host vessels to grafts of thyroid tissue. The first sprouts appeared on the third day, at which time some seemed to penetrate the tumor grafts a short distance. Within 1, 2, 3, and 4 days, respectively, 0, 8, 52, and 80 percent of the 25 grafts placed adjacent to nerves were vascularized. During the next few days the sprouts from the hosts produced a rich network of vessels throughout the grafts.

In most of the grafts from large tumors, the graft vessels did not seem to survive, whereas in many of the grafts from small tumors they did. The vessels of the grafts could not be seen until they became filled with host blood because no donor blood remained in them. During the first few days after vascularization, the surviving vessels of the grafts, which were engorged with host blood, extended far beyond the rich network of invading host vessels (see fig. 2). Surviving vessels in grafts of tumor tissue did not produce sprouts. At least, none were seen before the vessels in the grafts were obscured by the vessels growing from the hosts.

A few observations made on grafts of other rapid-growing adenocarcinomas and sarcomas indicated that the host reaction was similar to that described for the C3HBA adenocarcinoma.

The vascularization of grafts of tumor tissue was delayed when implants were so placed that more connective tissue than usual was left between the vessels of the grafts and those of the hosts. Within a day or two after

grafting, the nearby vessels became dilated and host leukocytes began to accumulate around the grafts. This reaction, which increased until the grafts became vascularized, may have been due to the growth of tumor grafts, for these grafts grew even before they were vascularized. Most of the grafts of the size used in these studies were vascularized by 10 days, even when placed far from host vessels. New sprouts from the vessels of the hosts grew from all sides into the areas of cellular reaction and into the grafts.

Formation of Sprouts in Injured Areas and Vascularization of Grafts Placed in Injured Areas

After nerves were cut, canalized sprouts were seen growing from the ends of the cut vessels of the nerves on the third day at the earliest. New vessels growing from the cut vessels and anastomosing with nearby vessels also appeared at 3 days. If a nerve was cut and 5 days later it was cut again, a few sprouts were seen in 1 or 2 days.

To see whether grafts placed in previously injured areas would be vascularized sooner than those placed in normal areas, 15 grafts of tumor tissue were implanted on previously injured beds. These grafts were vascularized 1 or 2 days earlier, on the average, than grafts on normal beds, but they did not seem to become well-defined masses of tissue with good circulation throughout any sooner. Eight grafts of young thyroid tissue and eight grafts of adult thyroid tissue were placed close to where sprouts were growing from vessels in previously injured nerves. Some grafts were vascularized in 1 or 2 days and some were never vascularized. The time of vascularization seemed to depend mainly on the distance between the graft and the host vessels. Sprouts did not grow from the vessels in these grafts any sooner than from the vessels in grafts placed on normal beds.

Discussion

Graft Vessels

The original vessels in grafts of thyroid tissue survived and produced sprouts, whereas the vessels in grafts of tumor tissue survived only in some grafts and did not produce sprouts.

Vessels in grafts of normal tissues.—Most accounts in the literature assign the active role in the establishment of connections between graft and host vessels to the vessels of the host alone. This is true whether these accounts are based on studies of histologic sections or on studies of grafts observed in transparent chambers. It is clear from the observations reported here and from those of Williams (*2*) that the vessels in grafts of normal tissue may produce an abundance of sprouts. Although, under the conditions of the present experiments, these sprouts were not effective in establishing connections with host vessels, it seems likely that they would be effective if the grafts were placed somewhere other than in a layer of avascular connective tissue.

That the vessels growing into connective tissue are oriented in their growth like the cells and fibers of the connective tissue has long been

recognized (*9*). In the present study the orientation of the elements within the connective tissue seemed also to determine the sides of the grafts from which the sprouts could arise. No sprouts grew on the sides of the grafts toward or away from the nerves. They grew only where, as they emerged from the surface of the grafts, they could immediately grow parallel to the direction in which the cells of the connective tissue were oriented. Surprisingly, vessels connecting the vessels of the grafts with those of the hosts formed on the sides of the grafts toward the nerves where no sprouts were seen. This suggests that solid threads of endothelium, the first stage in the formation of new sprouts, can grow where canalized sprouts cannot form. It suggests further that the solid threads forming at this site will not canalize unless they come in contact with other vessels.

Vessels in grafts of tumor tissue.—Some of the vessels in grafts of the C3HBA mammary adenocarcinoma survived, but they did not produce sprouts. This is similar to a finding of Browning on the survival of the vessels in tumor homografts placed in the anterior chamber of the eyes of mice (*10*). However, he did not observe similar survival of vessels in tumor isografts.

Relatively anoxic conditions were no doubt created by the growth of tumors without vascularization. Such anoxia, although it did not prevent the tumors from growing, may have prevented most vessels from surviving and may have prevented any surviving ones from forming sprouts.

Host Vessels

No sprouts grew from host vessels adjacent to grafts of normal tissue, whereas a rich network of sprouts grew from vessels adjacent to grafts of tumor tissue. A similar difference between normal and neoplastic tissues in their capacity to stimulate the growth of host vessels has been reported previously by Ide, Baker, and Warren (*3*) and by Algire and Chalkley (*4*). Not all tumors, however, stimulate host vessels. Williams (*11*) and Greene (*12*) have found tumors that do not cause new host vessels to form. Also, in this laboratory lymphoma #2 (*13*) was observed to spread out into a layer over the chamber area without affecting the host vessels. Only after the layer of lymphoma cells became relatively thick did new vessels grow from the host.

The capacity of grafts to alter the surrounding connective tissue so sprouts can penetrate it may be either qualitatively or quantitatively different from the capacity to cause new vessels to grow. Nonvascularized thyroid grafts caused rich networks of vessels to develop in the muscle layer adjacent to them, but no sprouts grew from the networks toward the grafts. Presumably sprouts did not grow because the connective tissue was not altered and the original orientation of the connective-tissue elements prevented growth except parallel to the plane of the muscle layer. Perhaps the reason thyroid grafts caused networks of vessels to form in the muscle layer but not in adjacent nerves is that the connective tissue around the nerve vessels is so oriented that there is no direction in

which the new vessels can grow. Cogan (*14*) found that an injury in the corneal tissue had little effect on nearby vessels unless the intervening corneal tissue had first been altered. Tumor tissue, it was found in the present observations, unlike the thyroid tissue, caused new sprouts to grow from host vessels regardless of the original orientation of the connective-tissue elements.

Interval before Sprouts Formed

An interval of 3 days elapsed after stimulation of vessels before canalized sprouts or new vessels, anastomosing with other vessels, were formed. This held true whether the vessels were in grafts, in cut nerves, or near grafts of tumor tissue. In a previous paper from this laboratory (*4*), it was stated that new vessels did not form until the 6th day after wounds were made in the fat and muscle layers of the skin. Although it was reported that the number of vessels increased during the 3rd to 5th days, this increase was attributed to the opening of collateral vessels. It seems probable, however, that this reported increase was in part due to the formation of new vessels, since, according to the present observations, some new vessels formed in 3 days. New vessels are easier to detect in cut nerves than in wounds in the muscle and fat layers because so few vessels are originally present in the nerves.

Three days is, in the present study, the shortest interval after stimulation before sprouts form. It is also the interval usually reported in the literature (*15*). Yet Williams (*2*) found that the vessels in a graft of omentum placed in a transparent chamber in the ear of a rabbit produced new canalized vessels or sprouts in 1 or 2 days, as did the vessels of the host bed. Such a short interval before new sprouts formed was observed in the present study only when the vessels were in an area that had been injured previously and that was presumably hyperplastic. However, Williams states that the bed on which the omental grafts were placed was stable. Therefore, it was presumably not hyperplastic. The shortness of the interval before both graft and host vessels produced new sprouts might be due to the condition of the connective tissue; or it might be due to the closeness of the vessels of the graft to those of the host, for in the present observations on thyroid grafts placed less than 50 μ from host vessels, the connecting vessels formed in some cases before sprouts grew from the graft vessels.

Sprouts grew from the vessels in grafts of young tissue sooner than from the vessels in grafts of adult tissue. The endothelium of the young tissue may have been more active. On the other hand, perhaps the young tissue altered the host connective tissue more than did adult tissue so that the sprouts could grow more rapidly.

Summary and Conclusions

The role of the graft and host vessels in the vascularization of grafts of thyroid and of mammary adenocarcinoma tissue was studied. Micro-

scopic observations were made on grafts placed in transparent chambers in the skin of mice.

The original vessels in grafts of thyroid tissue survived and provided most of the vessels of the grafts. These surviving vessels also were probably active in establishing connections between graft and host vessels. In tumor grafts, only a few of the original vessels survived and these vessels produced no new sprouts.

The host vessels adjacent to grafts of thyroid tissue seemed to remain unchanged and to contribute little to the vascularization of the grafts. On the contrary, the host vessels adjacent to grafts of tumor tissue produced, in each case, a rich network of vessels which provided almost all the vessels for the tumor graft. These findings confirm previous reports in the literature that grafts of tumor tissue may stimulate host vessels more than do grafts of normal tissue.

The vascularization of grafts of thyroid tissue was greatly affected by the original orientation of the elements of the host connective tissue. This orientation determined where vessel sprouts could arise and in what direction they could grow.

Bibliography

(1) Algire, G. H.: Initial host reaction to the graft. *In* Proceedings: Tissue Transplantation Conference. J. Nat. Cancer Inst. 14: 687–688, 1953.

(2) Williams, R. G.: The fate of minute blood vessels in omentum transplanted as autografts to the rabbit's ear. Anat. Rec. 116: 495–505, 1953.

(3) Ide, A. G., Baker, N. H., and Warren, S. L.: Vascularization of the Brown-Pearce rabbit epithelioma transplant as seen in the transparent ear chamber. Am. J. Roentgenol. 42: 891–899, 1939.

(4) Algire, G. H., and Chalkley, H. W.: Vascular reactions of normal and malignant tissues in vivo. I. Vascular reactions of mice to wounds and to normal and neoplastic transplants. J. Nat. Cancer Inst. 6: 73–85, 1945.

(5) Algire, G. H., and Legallais, F. Y.: Recent developments in the transparent-chamber technique as adapted to the mouse. J. Nat. Cancer Inst. 10: 225–253, 1949.

(6) Merwin, R. M., and Hill, E. L.: Fate of vascularized and nonvascularized subcutaneous homografts in mice. J. Nat. Cancer Inst. 14: 819–839, 1954.

(7) Barrett, M. K., and Morgan, W. C.: A maternal influence on the growth rate of a transplantable tumor in hybrid mice. J. Nat. Cancer Inst. 10: 81–88, 1949.

(8) The Committee on Standardized Nomenclature for Inbred Strains of Mice: Standardized nomenclature for inbred strains of mice. Cancer Res. 12: 602–613, 1952.

(9) Arnold, J.: Experimentelle Untersuchungen über die Entwicklung der Blutcapillaren. II. Die Entwicklung der Capillaren bei der Keratitis vasculosa. Arch. path. Anat. 54: 1–30, 1872.

(10) Browning, H. C.: Heterologous and homologous growth of transplants during the course of development of spontaneous mammary tumors in C3H mice. J. Nat. Cancer Inst. 8: 173–189, 1948.

(11) Williams, R. G.: The vascularity of normal and neoplastic grafts *in vivo*. Cancer Res. 11: 139–144, 1951.

(12) Greene, H. S. N.: The transplantation of human brain tumors to the brains of laboratory animals. Cancer Res. 13: 422–426, 1953.

(*13*) SHELTON, E.: Origin and behavior of two transplantable lymphomas induced by X radiation. J. Nat. Cancer Inst. 12: 1203–1223, 1952.

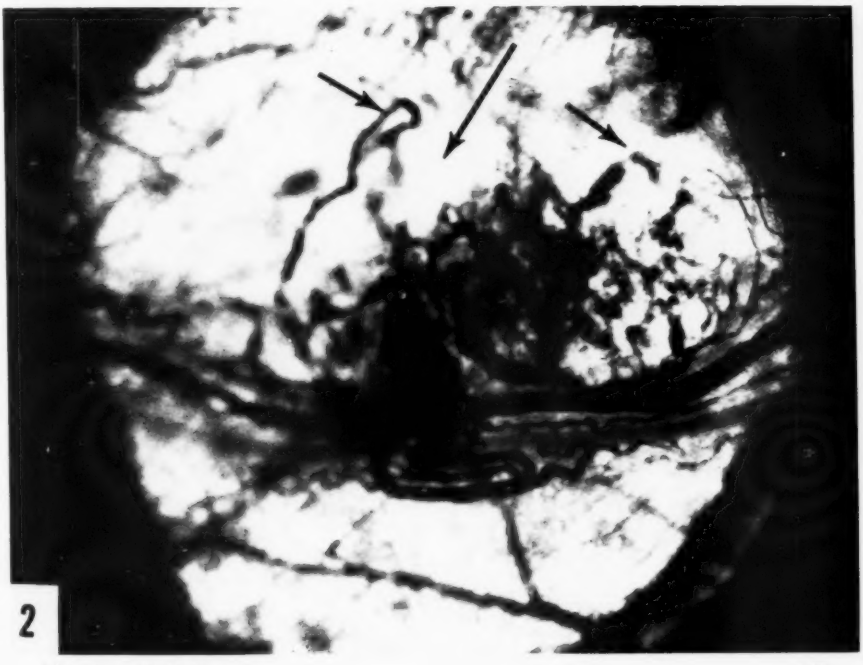
(*14*) COGAN, D. G.: Vascularization of the cornea, its experimental induction by small lesions and a new theory of its pathogenesis. Arch. Ophth. 41: 406–416, 1949.

(*15*) CAMERON, G. R.: Pathology of the Cell. Springfield, Ill., Charles C Thomas, 1952, 840 pp.

PLATE 6

FIGURE 1.—Graft of thyroid tissue from a mouse 1 day old, 6 days after grafting. No sprouts arise from the sides of the graft toward or away from the nerve. The position of the nerve is indicated by the large vein (marked by an *arrow*) that accompanies the nerve. All the sprouts growing from the vessels of the graft are parallel to the host nerve. The connecting vessels are out of focus. After the circulation in the graft became more rapid the vessels, which are here engorged with host blood, became narrower. × 100

FIGURE 2.—Piece of mammary adenocarcinoma tissue 4 days after transplantation. An unvascularized area of the graft is marked by a *long arrow*. The vessels marked by *short arrows* are graft vessels that survived and were engorged with host blood on the 2nd day. A rich network of vessels can be seen growing from the vessels in the host nerve. × 50



386036—56——5

The Influence of Sex on Isologous Skin Grafting in the Mouse [1]

RICHMOND T. PREHN *and* JOAN M. MAIN,[2] *National Cancer Institute,*[3] *Bethesda, Maryland*

The report of Eichwald (*1*) that isologous (*i.e.*, within one inbred strain) skin grafts were often rejected if the donor was male and the recipient female led us to investigate whether or not our own animals would exhibit a similar phenomenon.

Four strains were available for investigation: BALB/cAn, C3H/He, C57BL, and DBA/2. The animals varied from 3 to 6 months of age. Males were paired with females of the same strain, and skin grafts were exchanged between the members of each pair. The method of grafting (full thickness 2 × 3 cm.) has been described (*2*). The results are shown in table 1.

TABLE 1.—*Results of reciprocal skin grafts between male and female mice**

Strain	♂ graft, ♀ recipient		♀ graft, ♂ recipient	
	No. takes	No. mice	No. takes	No. mice
BALB/cAn	11	12	9	10
DBA/2	9	9	9	9
C3H/He	7	8	5	5
C57BL	0	6	7	7

* "Take" means a healed graft with hair growth 5 weeks after grafting. Mice which died prior to that are not reported.

It is apparent that our C57BL mice exhibited the same rejection of male grafts by female recipients as did the C57BL mice of Eichwald. The other three strains (which were not investigated by him) did not behave in this manner. In these three strains (BALB/cAn, DBA/2, and C3H/He) the sex of donor and recipient did not appear to influence tissue transplantation.

Since it appears probable that the sex influence is due to a hitherto undetected antigenic histocompatibility locus on the "y" chromosome,

[1] Received for publication March 2, 1956.
[2] With the technical assistance of Mr. Earl Waters.
[3] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

it should be possible to show a "second-set effect"—*i.e.*, second-set grafts of male skin on female mice should be destroyed at an accelerated rate as the result of acquired immunity in the female recipients. Eichwald has already demonstrated such an effect (*3*). Thus, the "second-set effect" might be a method of demonstrating antigens too weak to be observed by primary grafting alone.

Therefore, the strain BALB/cAn mice previously mentioned (both male and female) were treated as follows, 5 weeks after grafting: The growing graft from the donor of opposite sex was carefully and completely excised and replaced with a graft from a new donor of the same sex as the recipient. The animals were, by this means, "rested" for 2 weeks, during which time they were exposed only to skin grafts obtained from donors of their own sex. Each of the mice was then regrafted a third time with a fresh graft, this time taken from a new BALB/cAn strain donor of the opposite sex from the recipient. The animals were observed for 3 months. All of these grafts were successful (grafts on 10 female and 12 male recipients). Thus, an attempt to immunize the female mice against the males did not reveal an antigenic histocompatibility locus on the "y" chromosome in mice of the BALB/cAn strain.

It is concluded that the effect of sex on isologous skin grafts, while strong in strain C57BL, is relatively weak or actually lacking in strain BALB/cAn, C3H/He, and DBA/2.

References

(*1*) Eichwald, E. J., and Silmser, C. R.: Transplant. Bull. 2: 148–149, 1955.

(*2*) Prehn, R. T.: Tumors and hyperplastic nodules in transplanted mammary glands. J. Nat. Cancer Inst. 13: 859–871, 1953.

(*3*) Eichwald, E. J., and Silmser, C. R.: The genetics of skin grafting. New York Acad. Sc. In press.

Quantitative Studies on the Multiplication of Neoplastic Cells *in Vivo*. III. Metabolic Stability of Deoxypentose Nucleic Acid and the Use of Labeled Tumor Cells for the Measurement of Growth Curves [1,2]

László Révész, Arne Forssberg, *and* George Klein,[3] *Institute for Cell Research and Genetics and Institute of Radiophysics, Karolinska Institutet, Stockholm, Sweden*

A high degree of metabolic stability is often attributed to deoxypentose nucleic acid (DNA), at least in lower organisms (*1*). This implies that synthesis of DNA occurs only as preparation for cell division and there is no replacement of DNA in the resting cell. Cell division results in an equal partition of DNA between both daughter cells. By measuring the decrease of radioactivity in the DNA fraction of cells labeled prior to inoculation it was therefore possible to follow the growth curve of certain microorganisms. In higher organisms stability of DNA is more controversial (*2–4*), although results have been presented that favor a similar concept (*5,6*).

As pointed out by Hogness *et al.* (*7*), it is rather difficult to find mammalian systems that are favorable for such studies. Ascites tumors may represent an exception, however. Some long-established lines, such as the Ehrlich ascites tumor, consist of one major cell type and can be conveniently labeled with isotopes; furthermore all tumor cells are exposed in a similar fashion to the changes in the environmental conditions during the growth cycle.

Besides being a favorable system for studies on the stability of nucleic acids and proteins, there was another reason to undertake the present study on an ascites tumor; it concerns the general problem of measuring the growth of various experimental tumors. With solid tumors, this presents great difficulties (*8*) and is usually indirect and subject to various errors. The number of tumors convertible to the ascites form is limited and, among the convertible ones, there are several that do not grow exclusively in the free-cell form but include cell complexes. A number of established ascites tumors also infiltrate various solid tissues of the host to a large extent. Many difficulties might be circumvented by a method of isotope dilution, and an attempt was therefore made to devise experiments to test the extent to which such methods might be reliable. Another approach was made in a similar direction by Schleich and Wrba (*9*). These authors measured the total activity of P^{32}-labeled ascites-tumor

[1] Received for publication February 21, 1956.
[2] This work was supported by grants from the Swedish Cancer Society and the Wallenberg Foundation.
[3] Our sincere thanks are due to Miss G. Dreyfus and Mrs. U. Bogren-Sundwall for valuable technical assistance.

cells at various times after inoculation and compared it to the calculated cell number of the inoculated animals. Although their data show a certain rough parallelism between tumor growth and isotope dilution, it is not possible to draw any conclusions about the reliability of the method because of the indirect nature of the growth measurement and the lack of data on the distribution of radioactivity in DNA and other cell fractions. The present study is an attempt to measure isotope dilution after the inoculation of C^{14}-labeled Ehrlich ascites-tumor cells and to compare it to the curve of tumor-cell multiplication, determined by an independent quantitative method. The activity of various biochemical fractions was determined as a function of time after inoculation and correlated with the total number of free tumor cells in the peritoneal cavity.

Materials and Methods

Mice.—Heterozygous male mice of a body weight of 20 to 25 gm. were used for all experiments. They were kept on a standard pellet diet which, along with drinking water, was available *ad libitum* at all times.

Tumor.—The tetraploid line of the Ehrlich ascites tumor (*10*) was used. Routinely, it was kept by transfer of 0.2 ml. of ascitic fluid 7 to 10 days after the previous inoculation.

Labeled compounds.—Glycine-2-C^{14} and adenine-8-C^{14} were used, both with a specific activity of about 1 mC per m*M* delivered from The Radiochemical Centre, Amersham, England. They were dissolved and administered in saline at *p*H 7.0.

Labeling of ascites-tumor cells.—Small groups of mice (between 4 and 8 animals) were inoculated intraperitoneally with 2 million Ehrlich ascites-tumor cells. Labeled glycine or adenine was administered intraperitoneally after the 5th or 6th day following inoculation. The injections were repeated at regular intervals in the course of the following 2 days in doses of 1 μC in 0.1 ml. saline until a total dose of 5 μC was given. The animals were sacrificed 1 to 2 days after the last injection and their ascites was collected in ice-cold tubes and pooled. One aliquot was used to determine the concentration of cells in a Buerker hemocytometer. A second aliquot was subsequently diluted with Krebs-Ringer's-phosphate (containing 0.25% gelatin to prevent destructive swelling), and a total of 20×10^6 cells were injected intraperitoneally in volumes of 0.2 ml. into each of a group of 80 mice. This corresponds to 17.2×10^6 tumor cells on an average (*10*). Simultaneously, a third aliquot was used for determinations of radioactivity as described below.

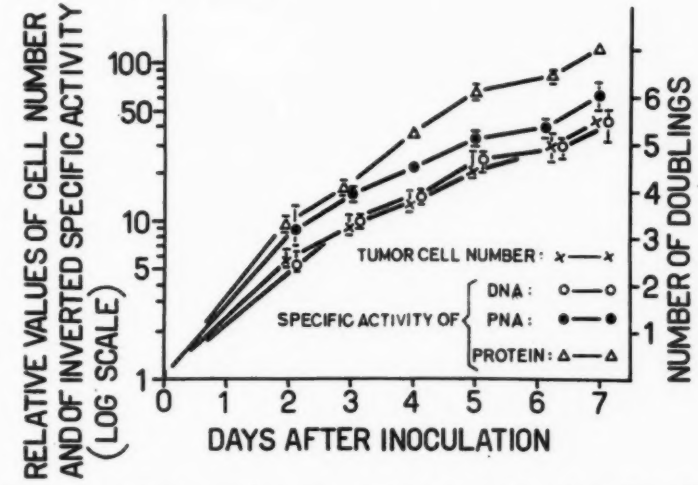
After the inoculation of the labeled ascites, between 10 and 20 mice were sacrificed daily from the 2nd until the 7th day. Larger numbers were used at the earlier than at the later stages in order to secure a sufficient quantity of ascitic fluid. The fluids of 6 or 15 mice were pooled and centrifuged and the radioactivity of the sediment and the supernatant were determined separately and expressed per unit of wet weight. The sediment was further assayed with regard to the activity of acid-soluble compounds, pentose nucleic acid (PNA), and DNA purines and,

in the case of glycine administration, proteins. Preparation and measurement of radioactivity were carried out in the same way as in a previous study (*11*). Simultaneously, the total ascitic fluid of four mice was collected quantitatively by rinsing with saline, and the total number of cells and the percentage of tumor cells were determined as described previously (*10*).

Results

Four series of experiments were carried out and the results are summarized in table 1. Glycine was used as the precursor in two and adenine in the remaining two. In both glycine series and in the first adenine series the inoculated cells were not washed, while in the second adenine series the cells were washed two times with physiologic NaCl, centrifuged at 1,500 × g for 10 minutes, and resuspended in saline after each washing. The latter experiment gave results somewhat different from those obtained with unwashed cells and they will therefore be presented separately.

The data obtained with unwashed cells appear in text-figure 1. Tumor cells, 17.2×10^6, were inoculated and the resulting growth curve is shown. To facilitate comparison with the growth curve, the specific activity of the nucleic acids and proteins was brought to the same scale by plotting the inverted values of specific activity against time after inoculation on a logarithmic scale where all initial values were taken as equal to 1. It will be seen that there is close agreement between the actual growth curve and the dilution of specific activity of DNA. In



TEXT-FIGURE 1.—Total number of free tumor cells and inverted specific activity of DNA, PNA, and proteins as a function of time after inoculation. All values were brought to a common scale (*left*) by taking the values corresponding to the inoculum as equal to 1. The three series glycine I, glycine II, and adenine I were utilized. Curves are drawn through the geometric mean of the values from the different series, each value representing a pool of ascites from 4 to 15 mice. The range of the values is indicated. The *right* scale shows the number of generations.

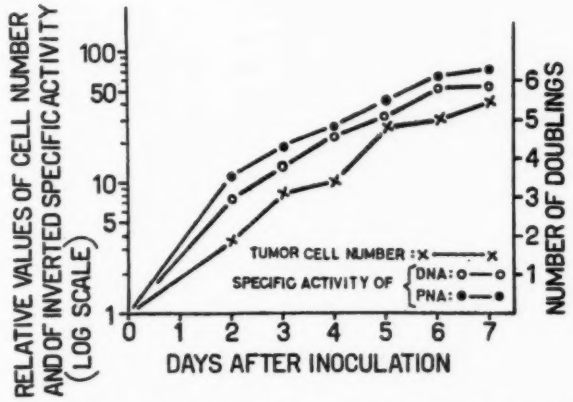
TABLE 1.—*Changes in the specific activity of various fractions and in cell number during the growth of Ehrlich ascites tumor at different times after the inoculation of labeled cells*

Series	Day after inoculation	Total* number of free tumor cells $\times 10^6$	Activity (C/min./mg.)†				Percent activity‡		Product of total cell number and specific activity		
			Wet cells	PNA purines	DNA purines	Proteins	Acid-soluble fraction	Supernatant	PNA $\times 10^9$	DNA $\times 10^9$	Proteins
Glycine I	0	17	162. 0	7, 430	6, 710	619. 0	5. 2	9. 0	126	114	105. 0
	2	98	19. 8	639	1, 070	65. 7	1. 2	0. 2	62	105	64. 4
	3	175	9. 7	535	660	34. 7	1. 2	1. 2	93	116	60. 7
	5	473	2. 9	217	274	10. 4	2. 0	3. 7	103	130	49. 2
	6	533	2. 3	192	221	7. 1	1. 3	1. 1	103	118	37. 8
	7	693	1. 3	101	139	4. 9	0. 9	2. 5	70	96	34. 0
Glycine II	0	17	131. 0	6, 770	6, 960	417. 0	6. 3	7. 1	115	118	77. 0
	2	90	14. 9	1, 040	1, 520	42. 7	1. 5	2. 9	93	137	38. 5
	3	160	4. 3	536	780	25. 0	0. 7	2. 4	85	125	40. 0
	4	195	1. 9	—	540	11. 6	1. 6	3. 9	—	105	22. 6
	5	330	1. 8	232	289	5. 9	1. 2	3. 8	76	95	19. 5
	6	398	1. 2	210	231	6. 0	1. 8	4. 7	83	92	23. 9
Adenine I	0	17	121. 0	31, 500	33, 300		7. 9	0. 7	536	566	
	2	106	20. 8	3, 820	6, 820		1. 0	0. 1	405	724	
	3	135	11. 7	2, 020	3, 500		1. 7	0. 4	273	473	
	4	265	6. 1	1, 460	2, 290		1. 2	1. 2	387	607	
	5	300	4. 1	960	1, 640		1. 4	0. 2	288	423	
	6	600	3. 5	735	1, 410		0. 8	2. 3	442	847	
	7	760	3. 1	640	1, 060		1. 8	1. 2	486	805	
Adenine II	0	17	284. 0	38, 300	38, 400		0. 9	0. 3	651	653	
	2	62	28. 4	3, 490	4, 830		0. 3	insignificant activities	216	299	
	3	145	12. 8	1, 955	2, 880		0. 6		286	418	
	4	175	—	1, 515	1, 639		—		265	286	
	5	480	5. 8	916	1, 173		0. 4		440	563	
	6	530	4. 6	585	722		0. 5		310	382	
	7	720	2. 9	515	699		0. 8		371	504	

*Mean of 4 mice. †Mean of 6 to 15 mice. ‡Expressed as percent of total cellular activity.

contrast, the curves calculated from the specific activity of PNA and protein deviate considerably from the growth curve—the latter to a larger extent than the former. The deviation increases progressively in the course of time in the case of the proteins, while with PNA there is only an initial deviation, occurring sometimes between the collection of the labeled cells for inoculation and the 2nd day of their subsequent growth in the new host. After the latter point of time, the PNA curve parallels closely both the growth curve and DNA. This is also apparent from the ratios of the specific activities of PNA and DNA (table 2). In the material used for inoculation, the value of this ratio was close to unity in all four experiments. In contrast, the samples collected during the subsequent tumor growth were consistently characterized by a lower value, which varied between comparatively narrow limits.

The data obtained with the washed cells (series adenine II) are shown in text-figure 2. In this case there was no close correspondence between the growth curve and the specific activities of either PNA or DNA, and the nucleic acids showed activities that were consistently lower than expected.



TEXT-FIGURE 2.—The same as text-figure 1 for the series adenine II.

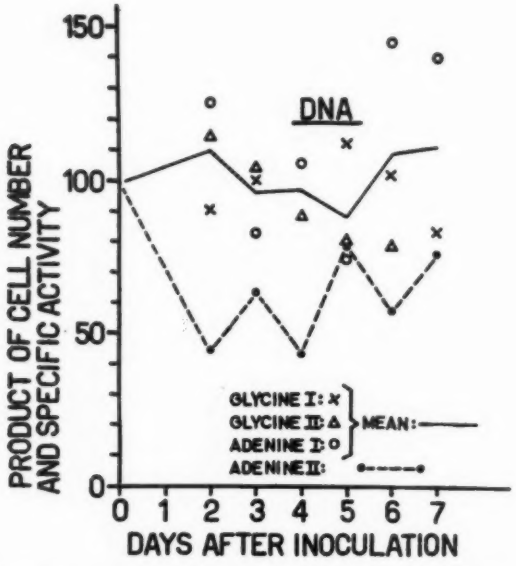
TABLE 2.—*Ratios of the specific activities of PNA:DNA in the inoculum and at various times after inoculation**

Inoculum		Days after inoculation	Glycine I	Glycine II	Adenine I	Adenine II
Series	Dose					
Glycine I	1. 11	2	0. 60	0. 69	0. 56	0. 72
Glycine II	0. 97	3	0. 81	0. 69	0. 58	0. 69
Adenine I	0. 95	4	—	—	0. 64	0. 93
Adenine II	1. 01	5	0. 79	0. 80	0. 59	0. 78
		6	0. 87	0. 91	0. 52	0. 81
		7	0. 73	—	0. 60	0. 75
Mean	1. 01		0. 76	0. 77	0. 58	0. 78

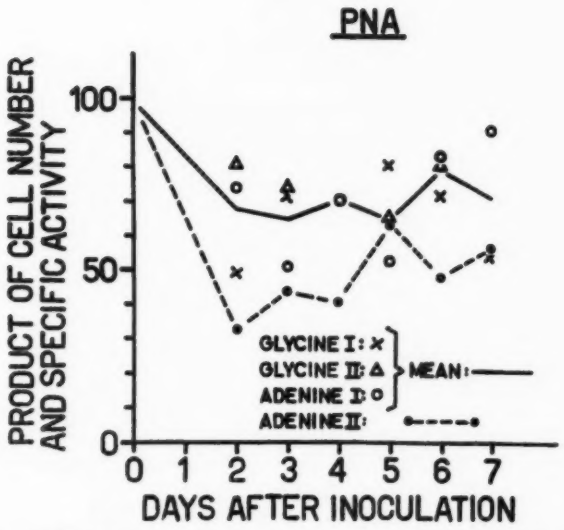
*Mean and standard error of all four experiments: 0.72±0.025.

To illustrate the variability between the different individual experiments and to compare the data with the hypothetical expectation of nucleic-acid stability, the products of specific activity and total cell number are given as a function of time for the various compounds in text-figures 3 to 5. The value of the product at zero time was taken as equal to 100, and the subsequent experimental points thus show the deviation in percent from the value expected in the hypothetical case of stability. With DNA (text-fig. 3), the mean of the three experiments with nonwashed cells shows only minor random variations around the expected constant product and does not deviate by more than ± 10 percent. This is only true about the mean; individual experimental values may vary up to 40 percent. The experiment with washed cells (adenine II) shows consistently low activity values. Corresponding curves for PNA (text-fig. 4) and protein (text-fig. 5) show initial fall followed by relative constancy of the product in the former case and a progressive fall of about 15 to 20 percent daily in the latter. The experiment with washed cells shows consistently low activities, even in the case of PNA.

Since the proteins contain the major part of radioactivity in the glycine series, it might be expected that the total cell activity would be determined primarily by the behavior of the proteins and would decrease with time more than can be expected from the cell multiplication rate. This was actually the case as shown in text-figure 6. In contrast, total cell activity in the adenine series is determined primarily by the nucleic acids and, therefore, shows a closer correspondence with the growth curve of the tumor cells.

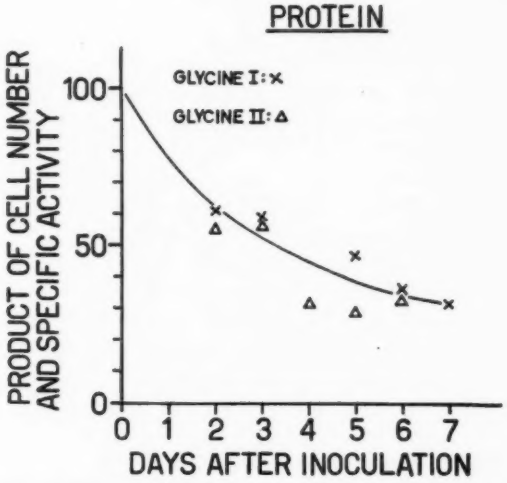


TEXT-FIGURE 3.—Products of total number of free tumor cells and specific activity of DNA fraction. The initial value is taken as equal to 100.

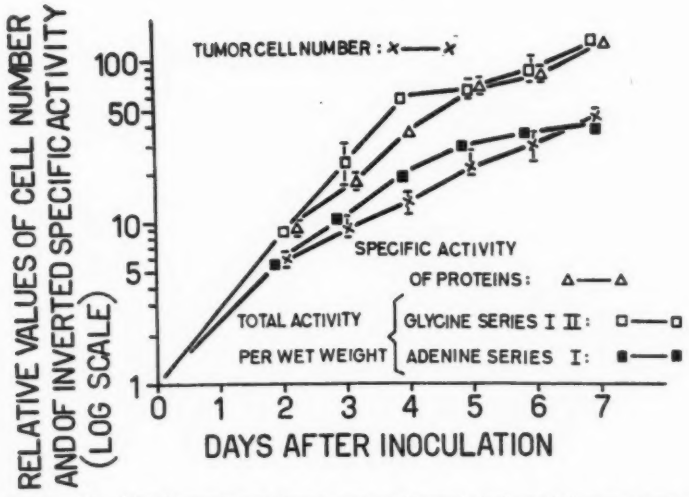


TEXT-FIGURE 4.—Products of total number of free tumor cells and specific activity of the PNA fraction. The initial value is taken as equal to 100.

Although activity is being lost from the proteins in the glycine series, the released isotope does not seem to be available for the synthesis of nucleic acids in any appreciable amount, since otherwise it would counteract the stability of the latter and would bring about progressively increasing specific activity during the later stages of growth. The proportion of radioactivity present in the acid-soluble fraction of the tumor



TEXT-FIGURE 5.—Products of total number of free tumor cells and specific activity of the protein fraction. The initial value is taken as equal to 100.



TEXT-FIGURE 6.—Total number of free tumor cells and their activity per wet weight in the two glycine series and in the series adenine I, respectively. The data have been treated in the same way as in text-figure 1. The specific activity of the protein fraction in the glycine series is also shown for comparison.

cells and in the ascitic supernatant was consistently low, of the order of a few percent (table 1). In the glycine series, these fractions nevertheless showed a somewhat higher proportion of activity than in the adenine series. This may be correlated with the above-mentioned loss of activity from protein in the former.

Discussion

The results of the present study favor the concept of a metabolically static state of deoxypentose nucleic acid in ascites-tumor cells. This is indicated by the close parallelism between the growth curve of labeled tumor cells in the peritoneal fluid and the geometric dilution of the specific activity in their DNA fraction. Such a concept implies that there is no breakdown of DNA between two cell divisions and that the original DNA molecules are divided equally between the daughter cells at each mitosis. Similar results were obtained by Hershey with *Escherichia coli* (*1*) and by Fujisawa and Sibatani (*6*) and Barton (*5*) with regenerating rat liver, but the results are at variance with the interpretation of Stevens *et al.* (*3*) of their experiments performed on intestinal mucosa and liver and with the studies of Barnum (*4*) on a transplanted mammary carcinoma.

The behavior of PNA presents a somewhat different picture. About 30 percent of activity is lost from this fraction during the time that elapses between inoculation and the first assay of activity 2 days later. Subsequently, the activity remains constant in the PNA fraction of the entire ascites system, however. This is reflected by the constant ratio between the specific activities of PNA and DNA and by the parallelism

between the growth curve and the inverted activity of PNA per cell. The initial loss could be interpreted in different ways. It is known that different cell fractions, such as nuclear and cytoplasmic fractions, are characterized by very different rates for incorporation of various isotopes into PNA (*12*). It is conceivable that some cell fraction, having a high turnover of PNA, is responsible for the initial loss of activity, while other fractions may be characterized by a high stability similar to that of DNA. Alternatively, there might be an initial loss of PNA molecules from the cells; Ledoux and Revell (*13*) found a decrease of the intracellular PNA content during the growth of the Landschütz ascites tumor.

It is of interest that the ratio of the specific activities of PNA:DNA remained constant during the entire growth period (with the exception of the first 2 days) and that the numerical value of this ratio did not depend on the nature of the precursor (glycine or adenine). In contrast to these findings, great differences were observed in the incorporation rate of adenine and glycine into PNA *versus* DNA in normal liver and other largely nondividing tissues. The differences were less or none when using rapidly growing tissues (*14*).

Deviating results were obtained in an experiment where washed cells were used for inoculation. The activity of both nucleic acids was consistently lower than could be expected on the basis of the growth curve. This is most probably explained as being due to an irreversible damage inflicted upon a certain proportion of tumor cells by washing, in conformity with the findings of Craigie (*15*) on the harmful effects of "physiologic" salt solutions upon populations of ascites-tumor cells. Presumably, the damaged cells could not participate in the continued growth of the tumor, and the size of the effective inoculum may have been smaller than the actual cell count. This would result in a higher isotope dilution than expected from the cell number. Assuming that this is the true mechanism, the data in text-figure 2 indicate that approximately 38 percent of the inoculated cells were lethally damaged. The fact that the growth curve does not differ appreciably from that of the unwashed inoculum (*cf.* text-fig. 1) does not invalidate this conclusion, since, due to the convergence of growth curves with different inoculums (*10*), initial differences of this order tend to level out rapidly and would probably not be detectable by the present methods.

The protein analyses indicated that the tumor cells are gradually losing activity from their proteins and at a higher rate than could be expected from the growth curve. This could be interpreted as evidence for a renewal of protein molecules, provided that increasing contamination with nonradioactive protein particles can be excluded. Such contamination does not seem to be impossible in view of the increasing protein content of the ascitic supernatant in the course of tumor growth (*13*) and the usually increasing erythrocyte content of ascites tumors. If the loss of activity in the protein fraction were due exclusively to contamination, however, a similar loss might be expected to occur in total cellular activity since the latter is primarily determined by the voluminous protein fraction. The

adenine series is of critical importance in this respect since the cellular proteins themselves are not labeled. An examination of text-figure 6 shows that total cellular activity falls in the glycine series in a manner similar to that of protein activity (as could be expected), while in the adenine series it is in rather good agreement with the growth curve of the tumor cells. The existing deviation may be interpreted as indicating the extent of contamination with inactive protein material. Since this deviation appears to be small, it may be concluded that the progressive loss of activity from the protein fraction in the glycine series cannot be accounted for by contamination and must represent a real exchange reaction. A similar result was obtained by Greenlees and LePage (*16*) on other mouse tumors. In microorganismal systems no renewal of proteins was found apart from synthesis in connection with cell division (*7,17*). It remains to be seen whether this difference is due to a difference in the nature of the biological material or in the labeled compound administered (S^{35}-sulfate and C^{14}-lactate with *E. coli*, C^{14}-glycine in the present study).[4]

The question arises as to what extent isotope-dilution methods can be used to measure the growth curve of tumor cells. It would appear that rather reliable growth curves can be obtained with the Ehrlich ascites tumor, using either C^{14}-labeled adenine or glycine as a tracer and measuring the specific activity of DNA as a function of time after inoculation. PNA would give a less correct picture and protein activity or total cell activity would be even less useful. It has to be pointed out that the present conclusions are valid only for the case of the *untreated* tumor. After treatment by radiation or chemotherapeutics, where part of the population is killed or damaged, isotope dilution may be upset by the incorporation of activity released from dead cells into the survivors. Studies in progress with artificial mixtures of labeled, lethally damaged, and unlabeled living cells indicate that such released activity is indeed utilized to a great extent by the cells that finally emerge in the treated tumor.

Summary

Ehrlich ascites-tumor cells labeled *in vivo* with glycine-2-C^{14} and adenine-8-C^{14} were inoculated intraperitoneally in known numbers. The subsequent increase in the number of free tumor cells and the decrease of the specific activity of the PNA, DNA, and protein fractions were determined at regular intervals. The inverted specific activity of DNA was in close agreement with the growth curve. This is in conformity with the assumption that DNA is metabolically stable and is synthesized only in relation to cell division. Its synthesis would involve a simple doubling, followed by equal subdivision between the daughter cells. PNA showed slightly different behavior: there was an initial loss of activity in excess of what could be expected from the growth curve. This loss occurred

[4] Preliminary experiments indicate that proteins labeled with S^{35}-methionine do not lose activity during the growth of the tetraploid Ehrlich ascites tumor used in the experiments described in this paper. The case is different for a hyperdiploid Ehrlich line. With this neoplasm activity is lost from S^{35}-methionine-labeled proteins to a considerable extent during the first 3 days after inoculation, while C^{14}-adenine-labeled DNA is stable. This shows that different results may be obtained with different precursors and with different tumor lines of similar (perhaps common) origin.

during the first 2 days of growth; during the later stages PNA showed a behavior similar to the DNA fraction. The proteins showed a progressive loss of activity as compared to the dilution expected from the growth curve. The results indicate that dilution of the specific activity of the DNA fraction of labeled tumor cells might be used to measure cell multiplication in the untreated Ehrlich ascites tumor.

References

(*1*) HERSHEY, A. D.: Conservation of nucleic acids during bacterial growth. J. Gen. Physiol. 38: 145–148, 1954.

(*2*) HEVESY, G.: Nucleic acid metabolism. *In* Advances in Biological and Medical Physics (Lawrence, J. H., and Hamilton, J. G., eds.). New York, Acad. Press, 1948, vol. 1, pp. 409–454.

(*3*) STEVENS, C. E., DAOUST, R., and LEBLOND, C. P.: Rate of synthesis of desoxyribonucleic acid and mitotic rate in liver and intestine. J. Biol. Chem. 202: 177–186, 1953.

(*4*) BARNUM, C. P., HUSEBY, R. A., and VERMUND, H.: A time study of the incorporation of radiophosphorus into the nucleic acids and other compounds of a transplanted mouse mammary carcinoma. Cancer Res. 13: 880–889, 1953.

(*5*) BARTON, A. D.: Evidence for the biochemical stability of desoxyribonucleic acid (DNA). (Abstract.) Fed. Proc. 13: 422, 1954.

(*6*) FUJISAWA, Y., and SIBATANI, A.: Is there any quantitative relationship between the synthesis and the breakdown of nucleic acids in living cells? Experientia 10: 178–180, 1954.

(*7*) HOGNESS, D. S., COHN, M., and MONOD, J.: Studies on the induced synthesis of β-galactosidase in *Escherichia coli:* The kinetics and mechanism of sulfur incorporation. Biochim. et biophys. acta. 16: 99–116, 1955.

(*8*) SCHREK, R.: Further quantitative methods for the study of transplantable tumors. The growth of R39 sarcoma and Brown-Pearce carcinoma. Am. J. Cancer 28: 345–363, 1936.

(*9*) SCHLEICH, A., and WRBA, H.: Anwendung von mit ^{32}P markierten Tumorzellen zur Bestimmung ihrer Teilungsgeschwindigkeit. Ztschr. Krebsforsch. 59: 543–545, 1953.

(*10*) KLEIN, G., and RÉVÉSZ, L.: Quantitative studies on the multiplication of neoplastic cells *in vivo.* I. Growth curves of the Ehrlich and MC1M ascites tumors. J. Nat. Cancer Inst. 14: 229–277, 1953.

(*11*) FORSSBERG, A., and KLEIN, G.: Studies on the effect of X-rays on the biochemistry and cellular composition of ascites tumors. II. Changes in the pattern of glycine-2-^{14}C incorporation during the first two hours after irradiation *in vivo.* Exper. Cell Res. 7: 480–497, 1954.

(*12*) SMELLIE, R. M. S.: The metabolism of the nucleic acids. *In* The Nucleic Acids: Chemistry and Biology (Chargaff, E., and Davidson, J. M., eds.). New York, Acad. Press, 1955, vol. 2, pp. 393–434.

(*13*) LEDOUX, L., and REVELL, S. H.: Action of ribonuclease on neoplastic growth. I. Chemical aspects of normal tumour growth: the Landschütz ascites tumour. Biochim. et biophys. acta 18: 416–426, 1955.

(*14*) BROWN, G. B., and ROLL, P. M.: Biosynthesis of nucleic acids. *In* The Nucleic Acids: Chemistry and Biology (Chargaff, E., and Davidson, J. M., eds.). New York, Acad. Press, 1955, vol. 2, pp. 342–392.

(*15*) CRAIGIE, J.: Survival and preservation of tumors in the frozen state. Adv. Cancer Res. 2: 197–228, 1954.

(*16*) GREENLEES, J., and LEPAGE, G. A.: Protein turnover in a study of host-tumor relationships. Cancer Res. 15: 256–262, 1955.

(*17*) ROTMANN, B., and SPIEGELMAN, S.: On the origin of the carbon in the induced synthesis β-galactosidase in Escherichia coli. J. Bact. 68: 419–429, 1954.

A Method of Freeze-Drying and Its Use in Histochemistry and Pathology [1]

M. S. BURSTONE, *National Institute of Dental Research,*[2] *Bethesda, Maryland*

During the past few years there has been renewed interest in freezing and drying for histologic and histochemical purposes. There is no doubt that under optimal conditions frozen-dried tissues exhibit excellent cytologic characteristics and do not show shrinkage artefacts so commonly seen with aqueous fixatives. Moreover, freezing and drying often represents the most desirable and in some cases the only type of "fixation" for enzyme histochemistry.

Despite these advantages, freezing and drying has not found widespread use for a number of reasons. One common belief is that only very small pieces of tissue (1 mm. thick or less) may be successfully frozen and dried without serious disruption of the architecture at the histologic level. This would seriously limit its use in histopathology where larger pieces of tissue should be used for adequate orientation. It also has been claimed that frozen-dried tissue is extremely hygroscopic and therefore must be embedded in the same vacuum chamber in which it has been dried. This factor would also tend to complicate the processing of any volume of tissue. In addition, many freezing and drying apparatuses utilize liquid-nitrogen traps and refrigeration which may require careful attention.

The present paper deals with the presentation of a system of freezing and drying which:

1) Will handle thick (5 mm.) slices of tissue in volumes adequate for a surgical pathology laboratory.
2) Will insure rapid handling and embedding of tissues.
3) Is capable of continuous operation over a period of weeks or months.

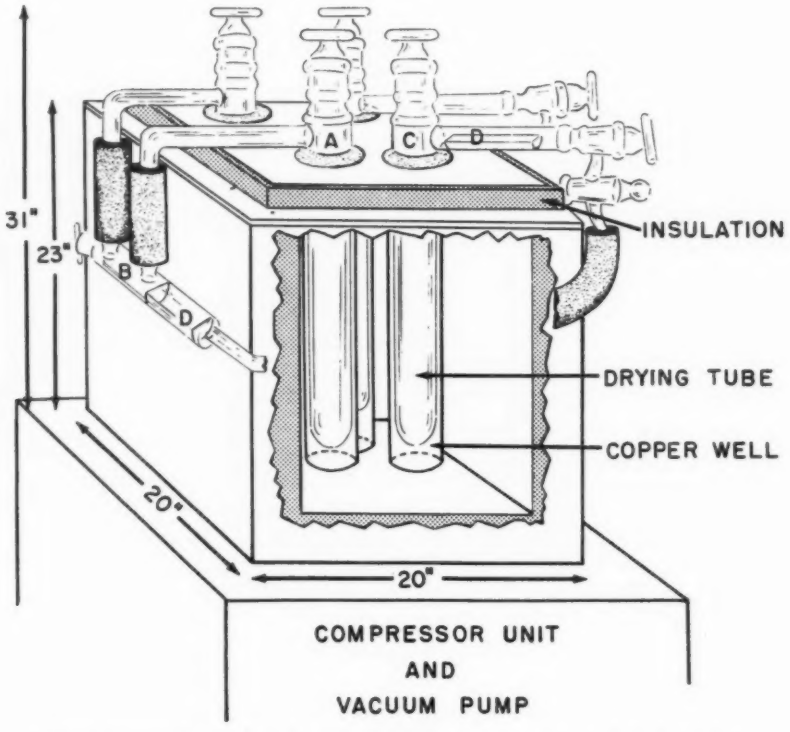
Design of the Freezing and Drying Apparatus

The freezing and drying apparatus, which has been in constant use in this laboratory for 2 years, is partly based upon the system developed by Gersh (*1*). It contains an electrically refrigerated chamber [3] using a low-temperature expansion valve for Freon-22, and a single stage ½ h.p. condensing unit [3] (fig. 1). A thermostatic control enables a temper-

[1] Received for publication February 24, 1956.
[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.
[3] Refrigerator made by Haskris Co., Chicago, Ill.

ature adjustment of −20 to −40° C. with no more than ±1° C. variation at any setting. The refrigerated box (fig. 1A) is permanently mounted on a stand which contains the compressor unit and vacuum pump (fig. 1B).

Two types of drying tubes are used (fig. 3 and text-fig. 1). Each has about a foot of usable space for tissue specimens. The first type is a simple tube about 2 inches in diameter (fig. 3A and text-fig. 1A), with a sidearm connection which is attached to the phosphorus-pentoxide manifold (fig. 3B and text-fig. 1B). The second type of drying tube is simpler in that the side arm contains the phosphorus-pentoxide manifold (fig. 3D and text-fig. 1D), and thus may be connected directly to a vacuum



TEXT-FIGURE 1.—Sectional view of refrigerated chamber and drying tubes.

pump. This tube may be installed in a deep freeze (capable of −28 to −30° C.) and thus constitutes the basis of a highly efficient freeze-dry system.

Each phosphorus-pentoxide manifold is fitted with a 2 mm. bore stopcock (fig. 3E). Although these are not absolutely necessary (provided that some means of bleeding air into the system is furnished), they are useful for attaching a vacuum gauge for periodic checks.

A large-bore 10 mm. stopcock (fig. 1C) is used to isolate the right and left sides of the machine. With the two sides isolated it was possible to connect another vacuum pump to one half of the system through stopcock D (fig. 1). This made possible an accurate comparison of drying rates in each side of the machine under different vacuums.

The Pirani gauge, figure 1E, though not absolutely necessary, is useful in observing the rate of evacuation of the system. It should be remembered that this type of gauge measures water vapor and thus is of little use when the system is filled with fresh tissue. The most versatile and least expensive gauge is the tilting McLeod gauge. During operation a vacuum of 10^{-3} to 10^{-4} mm. Hg is used, and a temperature of $-30°$ C. Although a vacuum within this range can be obtained with a good mechanical pump, great care must be taken in making hose connections and in sealing glass stoppers. Therefore it is recommended that a small oil diffusion pump be included. Statements to the effect that a diffusion pump is unnecessary may be misleading. Even though the requisite vacuum may be obtained with a good mechanical pump, continued operation of the system may result in slight contamination of the oil, imperceptible leaks in glass to rubber joints, or wear of vacuum stopcocks. Any of these factors may be difficult to correct, especially if many rubber connections are present.

Although the path of water molecules between the specimen and trap is greater than that of certain other freeze-dry systems, this does not appreciably increase the drying time. Therefore, it would appear that the mean free path of water molecules may be of secondary significance in the drying of small amounts of tissue.

Recent work has shown that the drying tissue itself offers the major resistance to water molecules, and for this reason the distance between the tissue and the trap need not be critical (*2*). Although the mean free path of a molecule of water at 10^{-3} mm. Hg is 5 cm., distances up to 20 cm. have been used in the system with little if any alteration in drying time.

A major factor in reduced drying rate is too great a drop in temperature of the tissue by loss of latent heat of evaporation. Since heat is best supplied to fairly large pieces of tissue by radiation, the glass tubes are surrounded by copper wells whose walls are ⅛-inch thick (text-fig. 1). Thus the drying tissues are supplied with radiant-heat energy. This type of system would seem to be more efficient than others in which the tissue temperature is determined by a flat heating plate. Although the tissue contacting the plate may be at the requisite temperature, the thermal conductivity of tissue is poor and the other side of the tissue is exposed to the cold of the liquid nitrogen or dry-ice trap.

Future attempts to increase the drying rate may involve more efficient systems for adequate heat exchange between the refrigerated chamber and the tissue.

Discussion of the temperature ranges used in drying is beyond the scope of this paper. Drying temperatures of -28 to $-30°$ C. are used in this laboratory. Preliminary studies with the system in use reveal no visible

histologic or histochemical difference between tissues dried over a range of −28 to −38° C. In industry, −30° C. is generally considered adequate for biological products (*3*).

Techniques of Freezing and Drying and Subsequent Processing

Freezing and Drying

The technique first described by Hoerr (*4*), which involves freezing tissues in isopentane cooled to about −160° C. by liquid nitrogen, is used in this laboratory. Three to 5 mm. slices of tissue are placed on aluminum foil and are dropped into a beaker of isopentane partly submerged in a Dewar flask of liquid nitrogen. The frozen tissues are quickly removed and placed in tubes submerged in liquid nitrogen. The tubes containing tissues are then transferred to the drying tube of the machine.

Six to 10 tubes of tissue may be placed in each of the drying tubes. With all 4 tubes thus filled the drying time is 5 to 7 days. However, if thinner slices of tissue are used the drying time is considerably less. If a few pieces of tissue approximately 1 mm. or so in thickness are used (as is usually recommended) the drying time is 6 to 12 hours. Comparisons of the effect of different vacuums upon the drying rate indicate that it is decreased above 10^{-3} mm. Hg, whereas it remains nearly constant between 10^{-3} and 10^{-4} mm. Hg. Above 10^{-3} mm. Hg drying rate is hindered. On the other hand below 10^{-3} to 10^{-4} the drying rate is not appreciably increased (at −30° C.). These observations appear to confirm the findings of Gersh and Stephenson (*1*).

Embedding and Subsequent Processing of Tissues

It is commonly believed that frozen-dried tissue is extremely hygroscopic and thus must be embedded in the apparatus without exposure to the atmosphere. Tests conducted in this laboratory have shown that frozen-dried tissues weighed on an analytical balance do not appreciably change in weight for periods up to 20 minutes. Moreover, there is no appreciable change in their characteristic chalky white appearance. When tissue removed from a freeze-dry machine turns a beefy red and appears wet after a minute or so, it probably indicates that the tissue was not dried and that upon exposure to room temperature the ice has melted.

Nevertheless, tissues may be embedded without removal from the machine by placing the frozen-tissue specimens on small glass boats which have previously been filled with paraffin and cooled with dry ice. The boats are then placed in the machine. After drying has been completed, the stopcock is closed and the entire drying-tube assembly is detached from the rubber-hose connection. It is then placed in an oven or water bath to melt the paraffin. When the paraffin melts the tissue will sink into it and be infiltrated. The assembly is then removed, air is allowed to enter, and the tissue is removed for embedding.

Although a heating coil may be built into one of the tubes for infiltration purposes in the machine, such a system necessitates shutting down the refrigeration unit, thus immobilizing the apparatus for drying. The author

believes that infiltration in the freeze-dry machine is in general a time-consuming and unnecessary procedure for most purposes. Degassing the paraffin is not recommended since some of the more volatile components may be lost and a paraffin of poor sectioning characteristics may result (*5*).

On the other hand, removing dried tissues from the freeze-dry machine and infiltrating them in a vacuum oven is a simple and efficient procedure. Such a procedure requires a vacuum oven (with a glass door or port) capable of maintaining a vacuum of less than 100 microns (fig. 2). With this type of oven one can simultaneously infiltrate a large number of tissues in small beakers of paraffin. It is important to remember not to apply a high vacuum unless the paraffin in the beakers is thoroughly melted in order to avoid frothing of the paraffin, which may push the tissue from the beaker. Infiltration time varies from 5 to 30 minutes. For a number of 5 mm. thick slices of tissue the latter infiltration time is necessary. Even though air may be removed from the tissue it still takes some time for paraffin to penetrate tissue interstices for adequate infiltration.

For some purposes, frozen-dried tissues may be embedded in celloidin. Another alternative involves double embedding in 2 percent collodion and then paraffin. The latter procedure may be of use in studying some of the more active enzyme systems such as kidney esterases. It is felt, however, that none of the present infiltration procedures is entirely satisfactory. Preliminary results appear to indicate that certain thermoplastic materials may be the most suitable infiltration media.

For the present, however, paraffin embedding, followed by sectioning and dry mounting on slides, is the procedure of choice. Successful dry mounting necessitates essentially wrinkle-free sections. Wrinkle-free sections of small blocks are easily obtained with a sliding microtome. In a few minutes several ribbons can usually be obtained. With larger blocks considerable skill and practice may be required to obtain essentially wrinkle-free sections. Successful dry mounting also necessitates absolutely clean slides. Slight traces of grease which might not affect ordinary mounting procedures will result in nonadherence of sections. After cleaning slides in alcohol, they may be placed on a metal rack and any final traces of grease removed by flaming them with a bunsen or blast burner. The clean slides are albuminized in the usual manner and the sections mounted with light finger pressure. Although it has been suggested that sections be "rolled on" with a finger, it has been found that such a procedure does not appreciably remove wrinkles and actually may tend to break up the section. After mounting the sections, the slides are placed on a thermostatically controlled hot plate (60° C. or the melting point of the paraffin) for about a minute. The hot plate should be placed at an angle so that the melted paraffin in the section will flow slightly and thus seal the section from the atmosphere. Mounted sections not so melted and sealed exhibit deterioration of enzyme systems (*6,7*). The slides are then refrigerated until needed.

Recently, the use of diacetin has been recommended for floating frozen-dried sections prior to mounting (*8*). Sections are flattened onto albumin-

ized slides by floating them on warm diacetin. The sections are blotted and allowed to dry overnight exposed to air. Preliminary tests with an esterase method (1-naphthyl acetate) has shown that this procedure may result in considerable and irregular loss of enzyme activity. In the case of succinic dehydrogenase, which can be demonstrated in dry-mounted kidney sections, complete inactivation follows the diacetin procedure. Aminopeptidase, which can be demonstrated in frozen-dried tissues, is also completely inactivated by the diacetin technique. Furthermore, diacetin-mounted sections frequently do not remain on the slides following petroleum-ether deparaffinization and subsequent acetone treatment, a procedure most useful for enzyme histochemistry. It appears somewhat inconsistent to freeze and dry tissues and then to float the sections on warm solvents of undetermined properties during which time the sections are also exposed to atmospheric moisture and oxygen. As previously indicated, smaller pieces of frozen-dried tissue may be easily sectioned wrinkle free provided that they are properly dried and infiltrated.

With certain plastic embedding materials, however, it may be possible to float sections upon certain liquids including water without appreciably affecting the sections.

For the more sensitive enzyme procedures, sections may be deparafinized in petroleum ether, followed by several changes of acetone (*9,10*). Xylene followed by absolute and 95 percent ethanol is satisfactory for most esterase and phosphatase procedures, as well as for the demonstration of protein groups (*7*).

For some enzyme procedures it is advisable to coat the section with a collodion or polystyrene film. The sections are then run from 95 to 85 percent ethanol or acetone directly into an enzyme substrate mixture.

Frozen-dried sections exhibit more stable characteristics than their fresh-frozen counterpart. Because of this stability, they may be subjected to prolonged "blocking" or staining procedures. In fact, many enzyme procedures may be run on these sections for 15 or 20 hours if desired.

Application to Histochemistry, Pathology, and Tissue-Culture Research

It is apparent that the use of routine fixatives for histochemical studies represented a compromise if not equivocal procedure. Although the exact details of tissue changes which occur during freezing and drying are not completely known, the fact that various frozen and dried biological and pharmaceutical products retain their native protein and other characteristics would recommend this process as the optimal type of histochemical fixation (*3*).

Recently there have been several reviews of the histology and histochemistry of frozen-dried tissues (*1,11*). Much of the previous work has dealt with the application of freezing and drying to avoid or minimize diffusion and other fixation artefacts. Classic examples of this application include study of glycogen granules, preservation of microscopic gas bubbles, and preservation of thyroid celloid (*1*). Salivary-gland mucin

granules, for example, are excellently preserved (*12,13*) (fig. 4). Considerable work has been done on the application of frozen-dried material for the localization of inorganic ions, microincineration, autoradiography, and ultraviolet and X-ray microscopy (*1,11*).

With the development of certain chromogenic methods for protein groups (*14*), frozen-dried tissue has found further application. With these methods chemically fixed tissues exhibit a more or less diffuse over-all stain, while frozen-dried sections exhibit a distinct contrast between many stained elements (*7*). These differences in staining presumably reflect the availability of certain protein groups (fig. 5).

The application of freezing and drying to enzyme histochemistry is a relatively neglected field and appears to have been limited primarily to the demonstration of phosphatases (*15–17*).

In many instances freezing and drying is the best if not the only procedure for preserving some of the more sensitive enzyme systems. Recently some of these enzymes have been demonstrated in normal and pathologic tissues. These included esterase (*9*) and aminopeptidase (*10*). Esterase, which may be inactivated in fixed tissue, has been demonstrated in frozen-dried salivary-gland tissue as well as in tumors (figs. 6, 7, and 8). In addition to localization of aminopeptidase in normal tissues, the enzyme has also been found in the stroma of malignant tumors (*18*) (fig. 9). It is apparent that the application of freezing and drying in conjunction with histochemical procedures to problems in pathology represents a fruitful field. Frozen-dried sections do not exhibit the marked diffusion artefacts seen with fresh-frozen sections, and thus have a potentially wide application for use with a large number of enzyme methods (fig. 10).

Thin films of tissue culture as well as tissue culture grown in Gelfoam sponge[4] lend themselves to optimal fixation by freeze-drying and subsequent study with enzyme and other histochemical procedures (fig. 11). The possibilities of such studies have not received even cursory appraisal.

Summary

The design of a freeze-dry apparatus and techniques for processing tissue subsequent to drying are presented.

The machine is capable of continuous operation and will handle a volume of 3 to 5 mm. slices of tissue adequate for surgical pathology needs.

References

(*1*) Gersh, I., and Stephenson, J.: Freezing and drying of tissues for morphological and histochemical studies. *In* Biological Applications of Freezing and Drying (Harris, R. J. C., ed.). New York, Acad. Press, 1954, pp. 329–384.

(*2*) Jansen, M. T.: Testing the performance of a simple freeze-drying apparatus for histochemical purposes. Exper. Cell Res. 7: 318–326, 1954.

(*3*) Flosdorf, E. W.: The development of freeze-drying. *In* Biological Applications of Freezing and Drying (Harris, R. J. C., ed.). New York, Acad. Press, 1954, pp. 63–86.

[4] Furnished through the courtesy of Dr. Joseph Leighton, Laboratory of Pathology, National Cancer Institute.

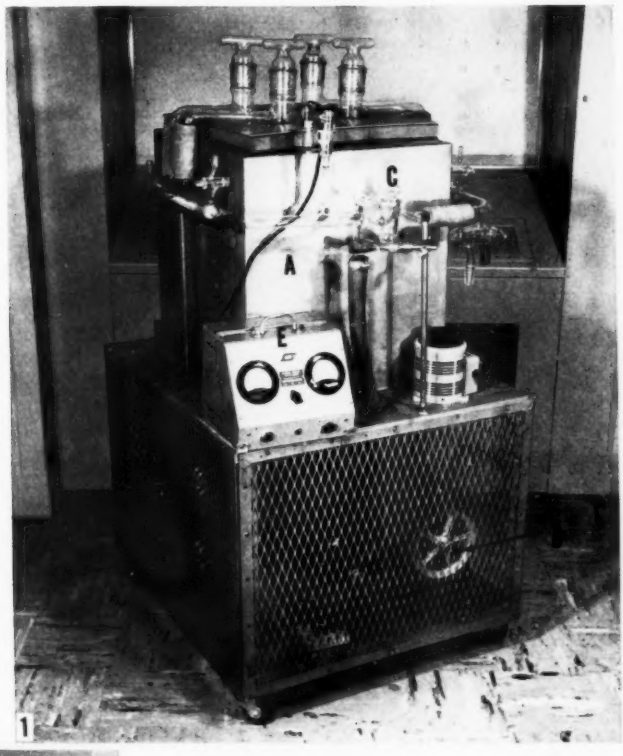
(4) HOERR, N. L.: Cytological studies by the Altmann-Gersh freezing-drying method. Anat. Rec. 65: 293–317, 1936.
(5) HARRIS, J. E., SLOANE, J. F., and KING, D. T.: New techniques in autoradiography. Nature, London, 166: 25–26, 1950.
(6) GÖMÖRI, G.: Microscopic Histochemistry: Principles and Practice. Chicago, Univ. Chicago Press, 1952.
(7) BURSTONE, M. S.: Unpublished data.
(8) FREED, J. J.: Freeze-drying technics in cytology and cytochemistry. Lab. Invest. 4: 106–122, 1955.
(9) BURSTONE, M. S.: Esterase of the salivary glands. J. Histochem. & Cytochem. 4: 130–139, 1956.
(10) BURSTONE, M. S., and FOLK, J. E.: Histochemical demonstration of aminopeptidase. J. Histochem. & Cytochem. In press.
(11) BELL, L. G. E.: The application of freezing and drying techniques in cytology. *In* International Review of Cytology (Bourne, G. H., and Danielli, F. J., eds.). New York, Acad. Press, 1952, vol. I, pp. 35–63.
(12) BURSTONE, M. S.: A histochemical study of normal and irradiated salivary gland tissue in the mouse. Anat. Rec. 115: 543–557, 1953.
(13) ———: A cytologic study of salivary glands of the mouse tongue. J. Dent. Res. 32: 126–132, 1953.
(14) ———: An evaluation of histochemical methods for protein groups. J. Histochem. & Cytochem. 3: 32–49, 1955.
(15) DEANE, H. W., and DEMPSEY, E. W.: The localization of phosphatases in the Golgi region of intestinal and other epithelial cells. Anat. Rec. 93: 401–417, 1945.
(16) WANG, K. J., and GROSSMAN, M. I.: Histochemical study of alkaline phosphatase in tissue prepared by the freezing-drying method. Anat. Rec. 104: 79–87, 1949.
(17) DANIELLI, J. F.: Cytochemistry: A Critical Approach. New York, Wiley, 1953, p. 139.
(18) BURSTONE, M. S.: Histochemical demonstration of proteolytic activity in human neoplasms. J. Nat. Cancer Inst. 16: 1149–1161, 1956.

The photomicrographs were taken by Mr. John McGuire. The photographs of the freeze-dry system were taken by Mr. S. Silverman.

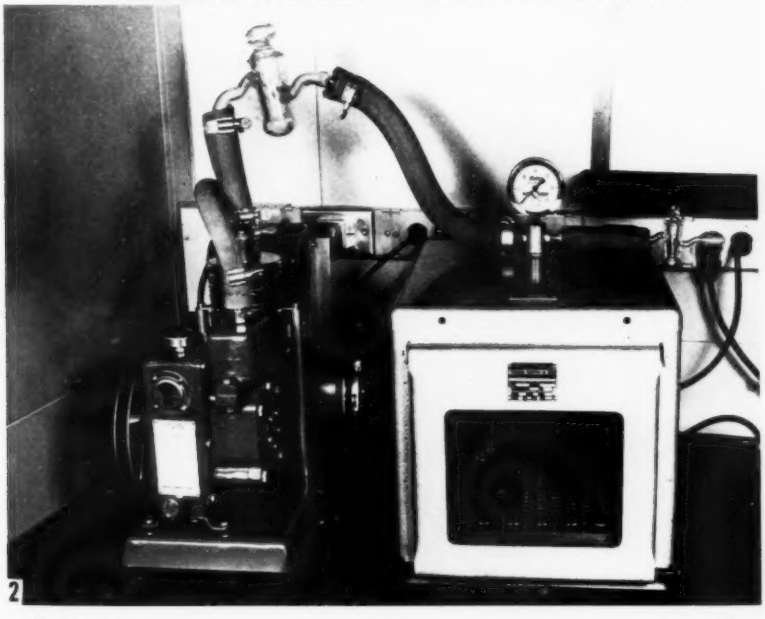
PLATE 7

FIGURE 1.—Freeze-dry machine showing:
A. Refrigerated chamber;
B. Vacuum pump and compressor unit;
C. Vacuum gauge.

FIGURE 2.—Vacuum oven for infiltration.



1



2

386036—56——7

PLATE 8

FIGURE 3.—Details of glass drying tubes showing two types of drying manifolds: B and D.

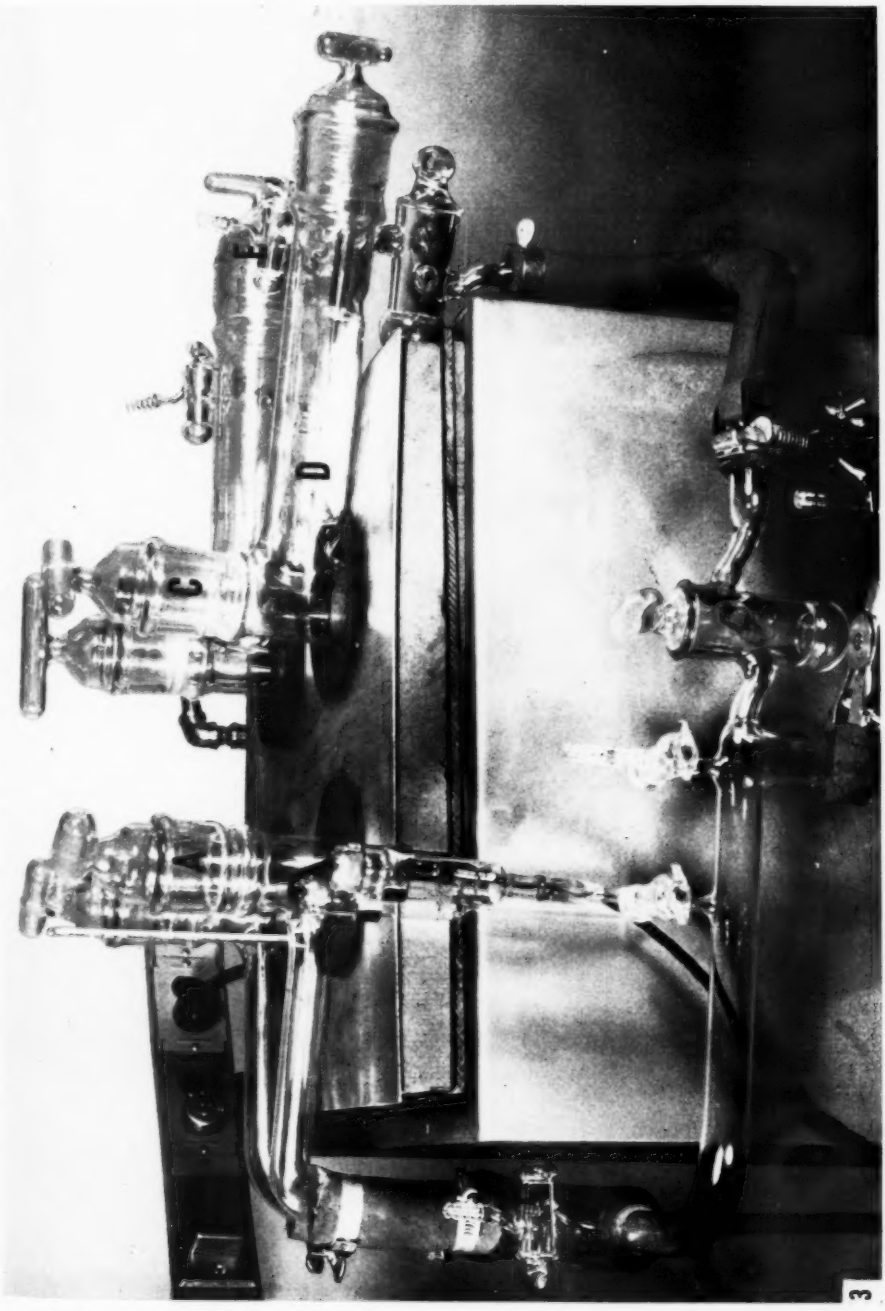

C
D
V
3

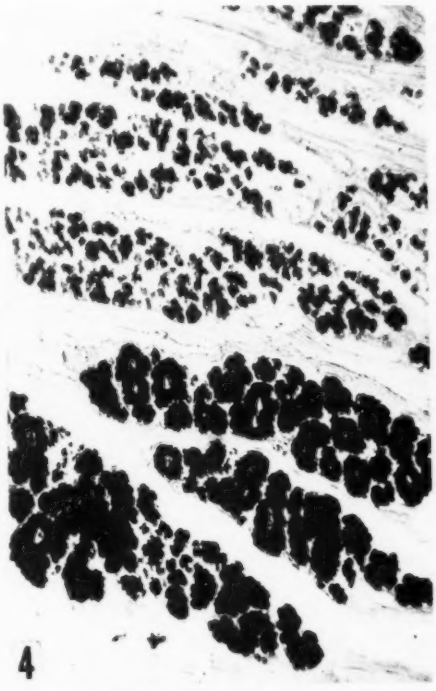
PLATE 9

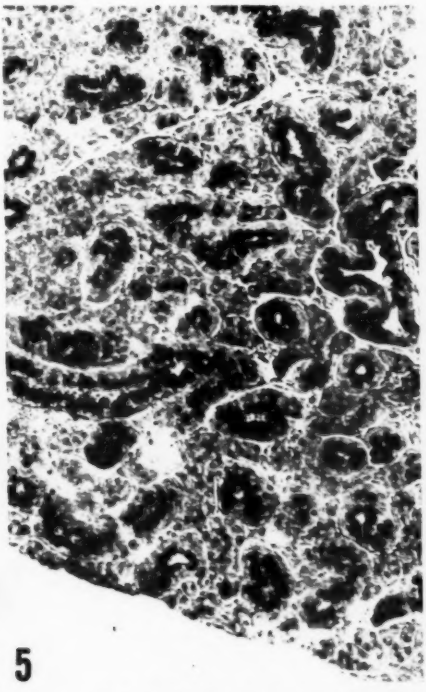
FIGURE 4.—Frozen-dried mouse tongue showing lingual glands, periodic acid-Schiff method. Both mucous and serous mucins stain and exhibit a granular pattern (*13*). × 140

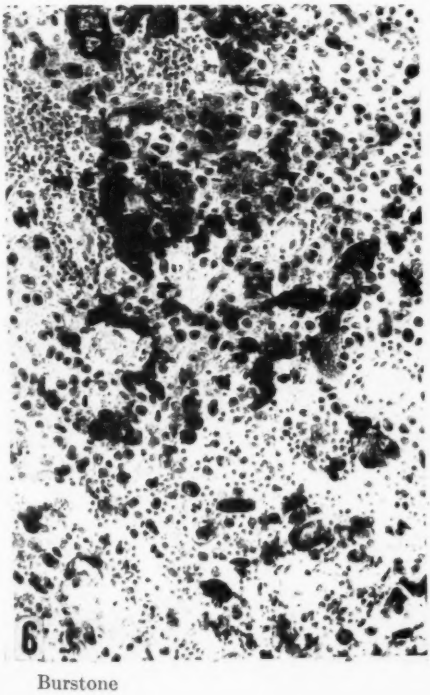
FIGURE 5.—Frozen-dried mouse submaxillary gland, ninhydrin-Schiff method. Mucoid cells stain intensely. × 140

FIGURE 6.—Esterase in tumor cells of a poorly differentiated lung tumor. × 140

FIGURE 7.—Similar area stained with hematoxylin and eosin. × 140


4


5


6

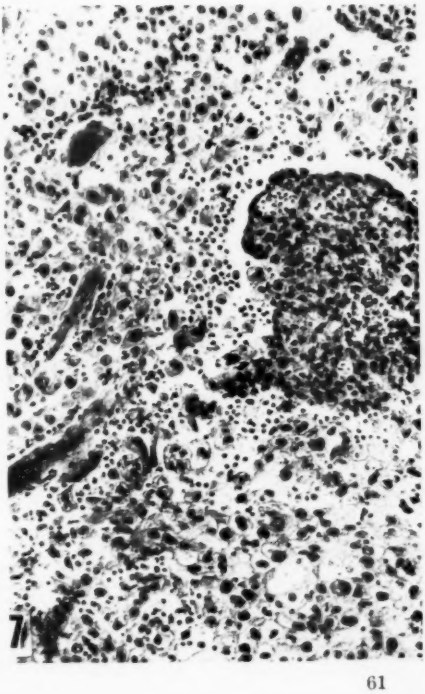

7

PLATE 10

FIGURE 8.—Esterase activity in ducts of human submaxillary gland. Naphthol AS-acetate procedure (*9*). × 110

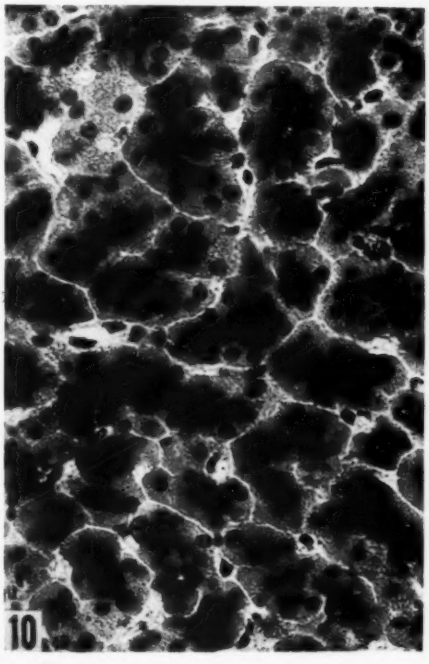
FIGURE 9.—Aminopeptidase activity in connective-tissue stroma of an epidermoid carcinoma. × 140

FIGURE 10.—Esterase activity in frozen-dried rat pancreas. The enzyme activity is associated with zymogen granules. × 415

FIGURE 11.—Frozen-dried tissue culture of HeLa cells, periodic acid-Schiff-hematoxylin. × 140

8

9


10

11

Some Biochemical Differences in Normal and Tumor Tissue (Sarcoma 180) of Mice: Amino Acid Composition [1,2]

M. N. MICKELSON *and* LOUIS BARVICK, *Chemistry Division, Midwest Research Institute, Kansas City, Missouri*

One of the theories for effective cancer chemotherapy is based on the existence of quantitative biochemical differences between normal and tumor tissue. Knowledge about differences in content of an essential vitamin or of amino acid, enzyme, or nucleic acid components might provide a basis for more effective exploitation of metabolite antagonists. Inhibition by an antimetabolite depends on the concentration ratio between the metabolite and the antagonist. If the metabolite in question is in lower concentration in the tumor tissue than in the normal tissue, it is reasonable that a given concentration of antimetabolite would block the utilization of that metabolite in the tumor to a greater extent than it would in the normal cells, thus causing less injury to the latter.

In the synthesis of anti-tumor agents, in progress in these laboratories, a number of amino acid analogues have already been synthesized. It was felt that information on the quantitative relationship between the amino acid composition of proteins in tumor compared with those of normal tissues might serve as a guide in the selection of amino acids for the preparation of more effective antagonists.

Studies similar to those in this report have been made with other animals and other tumors, using microbiological methods, *cf.* Sauberlich and Bauman (*1*) and Sassenrath and Greenberg (*2*). Others have used chromatographic methods—Roberts and Chao-T'E Li (*3*), Roberts and Frankel (*4,5*), and Kit and Awapara (*6*). Both techniques have also been used (*2*).

Sauberlich and Bauman analyzed samples of Flexner-Jobling carcinoma (a methylcholanthrene-induced sarcoma), two chemically induced hepatomas, and a spontaneous mammary fibrosarcoma for 18 amino acids (free plus those derived from hydrolysates) and compared them with those from normal tissues. They found the amounts of amino acids in the tumors to be very similar to those of normal rat tissues and to those of ordinary cuts of beef or pork. The mammary fibrosarcoma was unusual in that it contained large amounts of glycine and proline. They concluded

[1] Received for publication March 29, 1955.

[2] This investigation was aided in part by Research Grants C-802 and C-2479 from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service and in part by the Midwest Research Institute.

386036—56——8

that the gross amino acid composition of tumors was like that of most other animal tissues. Their results did not support the concept of Kögl and Erxleben (*7*) that tumors are characterized by large amounts of D-glutamic acid nor the results reported by Dunn, Feaver, and Murphy (*8*) that the arginine content of tumors was exceptionally high.

Sassenrath and Greenberg, working with Walker carcinosarcoma 256 in rats, used both chromatographic and microbiological methods, and found no change in the free-amino-acid profile that was characteristic for muscle, liver, and plasma in the tumor-bearing animal. However, they do not report results for the tumor tissue.

Kit and Awapara determined the free-amino-acid content of four lymphosarcomas, a transplantable methylcholanthrene-induced rat tumor, and normal lymphatic tissues of the mouse, rat, and rabbit. They found higher concentrations of alanine and glycine and lower concentrations of aspartic acid in the tumors than in the normal tissues.

Both increased and decreased content of some amino acids in the proteins of malignant compared to normal tissues have been reported with various tumors in rats, mice, and rabbits. The significance of the differences is still in question. However, it is imperative that there be biochemical differences between tumorous and nontumorous tissues to provide a basis for the chemotherapy of tumors. These differences may be in enzyme, amino acid, or vitamin composition or, in fact, any essential metabolite. This work deals with differences in amino acid composition of normal and tumor tissue (sarcoma 180) in mice.

The aforementioned theory is supported by some experimental evidence. Shapiro, Shils, and Dietrich (*9*) found that deoxypyridoxine, a vitamin B_6 antagonist, was carcinostatic against mouse mammary carcinoma No. 755. They found a lower concentration of vitamin B_6 in the tumorous tissues than in the majority of normal host tissues. Furthermore, they found that glutamic-aspartic transaminase, a pyridoxine-containing enzyme, was inhibited by deoxypyridoxine.

An investigation has been made of the amino acid (free amino acids plus those derived from hydrolysates) composition of mouse sarcoma 180 and the nontumorous tissues of tumor-bearing as well as non-tumor-bearing mice.

Methods

Strain BALB/c mice were used as tumor hosts. Sarcoma 180 tumors of 1 week's duration were removed from animals and cut into pieces approximately 2 $mm.^3$ in size and implanted in the axillary region by the usual trocar method. The implantation was done aseptically. The mice, 10 in each group, were allowed free access to Purina laboratory chow and water except in the case of the starved animals. They were deprived of food but allowed free access to water. On the 4th day of starvation 5 of the animals had died. The livers from the remaining 5 animals were removed and used for the amino acid analyses.

Microbiological analyses for methionine, phenylalanine, leucine, isoleucine, threonine, lysine, valine, arginine, histidine, serine, aspartic

acid, tryptophan, and tyrosine were made by the procedure of Stokes, Gunness, Dwyer, and Caswell (*10*). Aspartic acid and phenylalanine were determined with *Lactobacillus delbrueckii* LD5 (NRRL) and the remaining amino acids of this series with *Streptococcus faecalis* (NRRL).

Glutamic acid and cystine were determined with *Lactobacillus arabinosus* 17-5 by the procedure of Hac, Snell, and Williams (*11*).

Glycine and proline were determined with *Leuconostoc mesenteroides* (NRRL 1153) using the basal medium described by Barton-Wright (*12*).

All samples were run at three concentration levels and duplicate analyses were made. In none of the cases was appreciable "drift" apparent. Tumors (sarcoma 180) of 10 and 13 days' duration were analyzed for the 17 amino acids. Although a large spread in the age of tumors was desired, the harvesting of the tumors at 13 days was necessary because the mice were in their terminal stages. The tumors were carefully trimmed of necrotic and connective tissue.

Liver from these mice was chosen as the nontumorous tissue for comparison. The data in table 4 indicate liver to be a valid reference tissue for comparison with the tumor (*see* discussion). There was no quantitative basis for choosing one tissue over the other. In addition to livers from the tumor-bearing animals, those from normal (non-tumor-bearing) mice and mice that had been starved for 4 days were used. The livers were harvested at this time because the mice were in their terminal stages.

The pooled tissues from 10 animals of each group were placed on tared watch glasses and weighed. They were dried overnight at 65 to 70° C. at atmospheric pressure; the following day they were dried for 8 hours at the same temperature *in vacuo*. The dried tissues, after being weighed for moisture content, were finely pulverized. About 1.5 gm. of each sample were weighed into a small extraction thimble 10 × 50 mm. and extracted continuously with ethyl ether for 8 hours in a small Kutscher-Steudel type extractor for removal of lipide material.

Approximately 1 gm. of the dry, lipide-free tissue was hydrolyzed in a sealed glass ampoule with 10 ml. of HCl (10% by volume) for 10 hours at 125 to 127° C. in an autoclave. The hydrolysates were then neutralized to *p*H 6.8, filtered and made up to 50 ml. in a volumetric flask, and stored in the refrigerator under toluene.

In the case of tyrosine and tryptophan, 0.1 gm. samples of tissue were hydrolyzed in 5 ml. of 5 *N* NaOH in a covered 18 × 100 mm. test tube for 8 hours at 125 to 127° C. The hydrolysates were centrifuged to remove a bulky precipitate; this precipitate was then washed several times with water. The supernatant liquor and washings were neutralized and made to a volume of 25 ml. These hydrolysates were also kept in the refrigerator under toluene for analysis.

The total nitrogen content of each of the extracts was determined by a semimicro Kjeldahl procedure.

With a second series of animals, the amounts of the amino acids leucine, isoleucine, aspartic acid, histidine, methionine, valine, and tryptophan were determined in tumor tissue, liver, kidney, heart, spleen, brain, in-

testine, and muscle tissues from tumor-bearing as well as from non-tumor-bearing, normal animals. The tissues were all treated in the same manner as described above, with analyses made for moisture, lipide, and total nitrogen in the hydrolysates.

The sum of the nitrogen contents of the 17 amino acids for which analyses were carried out accounted for 70 to 87 percent of the total nitrogen in the hydrolysates as determined by the Kjeldahl method.

Results

Values obtained for the lipide, dry substance, and nitrogen content of the tumor and certain normal mouse tissues are shown in table 1. In the first part of the table are values from those animals whose tissues were analyzed for 17 amino acids; in the second part the values are for a series of normal tissues from normal and tumor-bearing mice that were analyzed for leucine, isoleucine, aspartic acid, histidine, methionine, valine, and tryptophan.

The lipide content of the liver tissue in the second series of tumor-bearing animals was considerably lower than that in the first group. In

TABLE 1.—*Fat, moisture, and nitrogen content of certain normal and tumorous tissues (sarcoma 180) of mice*

(10 animals in each group)

Tissue	Percentage dry matter	Percentage dry tissue		
		Fat	Total nitrogen	% Total N as amino N
Normal mouse liver	31. 0	17. 7	12. 78	82
Liver from mice with 10-day tumors	30. 2	25. 5	13. 63	87
Liver from mice with 13-day tumors	30. 0	17. 3	13. 83	86
Tumor tissue—10-day tumors	18. 9	18. 8	14. 20	69. 5
Tumor tissue—13-day tumors	19. 5	15. 6	13. 67	77
Liver from starved animals*	31. 0	24. 9	14. 41	75

Tissue	Percentage dry matter		Percentage dry tissue			
			Fat		Total nitrogen	
	Tumorous animals	Normal animals	Tumorous animals†	Normal animals	Tumorous animals	Normal animals
Brain	22. 3	22. 3	20. 0	37. 0	11. 45	12. 32
Heart	27. 7	22. 0	3. 50	17. 0	13. 44	13. 74
Intestine	24. 3	14. 7	5. 88	23. 0	12. 09	12. 09
Kidney	27. 8	28. 4	11. 60	25. 3	12. 52	14. 65
Liver	31. 0	31. 8	06. 95	16. 7	13. 06	10. 62
Muscle	26. 9	23. 0	6. 23	22. 3	12. 75	14. 07
Spleen	29. 0	25. 5	0. 45	12. 2	12. 81	13. 76
Tumor	18. 0	—	11. 6	—	13. 91	—

*Average mouse weight at start of experiment was 23 gm. and food consumption 4.3 gm. per mouse per day. After 1, 2, and 3 days of starvation the average weight of mice was 20.4, 18.3, and 16.5 gm., respectively. (Pooled tissue from 5 animals.)

†Average weight of mice at start was 24 gm.; 13 days after implantation 19.5 gm. Food consumption at start was 5.5 gm. per mouse per day, at the end 2.9 gm. per mouse per day.

fact, all of the tissues from the tumor-bearing animals were appreciably lower in lipide than were comparable tissues from nontumorous animals.

By means of microbiological-assay procedures the amino acid composition of mouse sarcoma 180 and certain normal tissues was determined. In the first experiment livers from normal and starved mice, and livers and tumors from tumor-bearing mice were analyzed for 17 amino acids. Results are expressed as percentage composition of dry, lipide-free tissue. Mice bearing tumors of 10 and 13 days' duration were used. Since no outstanding differences were noted in amino acid composition of animals with tumors of 10 and 13 days' duration, the data, except where noted, relate to animals with 13-day tumors. The results of this experiment are presented in table 2. Further comparisons are given in table 3, second column.

Nine of the amino acids were in lower concentration in the tumor tissue than in the nontumorous liver tissue from homologous animals (table 3).

In the case of phenylalanine, tryptophan and methionine, and tyrosine the differences were less than 10 percent. In the case of leucine, isoleucine, valine, histidine, and aspartic acid, the amounts ranged from 10 to 49 percent less in the tumor than in the liver. The greatest difference was in aspartic acid and histidine content, and in the first experiment the tumor contained half as much aspartic acid as the nontumorous livers (*see* table 3).

For five of the amino acids the content in the tumor was greater than in the liver. These were lysine, arginine, glutamic acid, proline, and glycine. The difference in content of these amino acids between liver and tumor tissue was small and perhaps of doubtful significance except for arginine. The 10th-day arginine sample showed a slight difference in the reverse direction (table 2). An increased content of arginine in rat fibrosarcoma was reported previously (*8*).

The content of threonine, cystine, and serine was substantially the same in liver and tumor.

The liver tissue from normal and starved mice (table 2) was analyzed to determine whether the liver tissue of starved animals might resemble that of liver from tumor-bearing animals. In general, the amino acid composition of liver from normal animals was not notably different from that of liver of starved animals. Arginine and serine were the only amino acids which varied significantly. Arginine was 34 percent higher in starved liver than in normal liver and 14.2 percent higher in tumor than the liver tissue from the 13-day tumor-bearing animals. The tumor value was slightly lower than liver in the animals with 10-day tumors. Serine was 13 percent higher in the normal liver than in the liver from starved animals. In table 3 are given the percentage differences of each of the seven amino acids which were lower in the tumor tissue than the nontumorous liver tissue from the two groups of animals. The values in table 3 show reasonable agreement in the two experiments except in the case of methionine.

TABLE 2.—*Content of 17 amino acids in liver and tumor tissue (sarcoma 180) of mice**

(5 animals in starvation group; 10 animals in all other groups)

Amino acids	Nontumorous animals		Tumorous animals			
	Normal liver	Starvation liver	10-Day tumor		13-Day tumor	
			Liver	Tumor	Liver	Tumor
Phenylalanine	7.08 ± .14†	6.87 ± .21	6.45 ± .04	6.13 ± .14	6.55 ± .06	6.36 ± .12
Methionine	1.90 ± .10	2.01 ± .32	2.06 ± .17	1.89 ± .072	1.88 ± .078	1.77 ± .143
Leucine	6.84 ± .055	7.28 ± .20	7.49 ± .24	6.34 ± .02	7.58 ± .65	6.83 ± .21
Isoleucine	4.30 ± .05	3.87 ± .10	4.49 ± .19	3.83 ± .05	4.30 ± .16	3.74 ± .06
Lysine	2.27 ± .038	2.63 ± .038	2.60 ± .009	2.76 ± .10	2.65 ± .03	2.77 ± .12
Threonine	3.69 ± .06	3.75 ± .15	3.73 ± .15	3.68 ± .04	3.61 ± .11	3.67 ± .09
Arginine	3.89 ± .11	5.22 ± .185	4.02 ± .05	3.76 ± .23	3.93 ± .22	4.49 ± .09
Valine	4.47 ± .26	4.34 ± .23	5.05 ± .21	4.36 ± .27	4.77 ± .18	4.24 ± .08
Histidine	2.54 ± .11	2.46 ± .014	2.50 ± .055	1.95 ± .075	2.59 ± .03	2.05 ± .064
Cystine	0.701 ± .07	0.566 ± .089	0.846 ± .045	0.754 ± .06	0.718 ± .057	0.702 ± .027
Aspartic acid	15.87 ± 3.16	13.88 ± .33	18.55 ± 1.88	9.38 ± 1.65	18.43 ± .67	9.26 ± 1.16
Serine	6.97 ± .35	6.17 ± .275	7.38 ± .44	6.86 ± .275	6.58 ± .18	6.53 ± .23
Glutamic acid	9.76 ± .16	9.50 ± .40	9.48 ± .48	10.76 ± .14	9.93 ± .27	10.74 ± .23
Tryptophan	1.21 ± .005	—	1.25 ± .012	1.04 ± .009	1.04 ± .017	0.99 ± .02
Tyrosine	7.55 ± .45	—	6.78 ± .22	7.29 ± .03	6.98 ± .34	6.80 ± .39
Proline	4.28 ± .148	4.59 ± .37	4.33 ± .245	4.72 ± .215	4.21 ± .195	4.51 ± .16
Glycine	4.06 ± .186	4.72 ± .182	4.25 ± .096	4.33 ± .163	4.17 ± .108	4.26 ± .096

*Percent of dry, lipide-free tissue.

†S.E.$=\sqrt{\frac{\Sigma(\bar{x})^2}{n(n-1)}}$.

TABLE 3.—*Comparison of amino acids in liver and tumor tissue expressed as percent difference from value for liver*

More in tumor than liver		Less in tumor than liver		
Amino acids	Percent	Amino acids	Percent	
			Expt. 1	Expt. 2
Lysine	4. 53	Phenylalanine	2. 90	—
Arginine	12. 5	Leucine	9. 90	11. 3
Glutamic acid	7. 60	Isoleucine	13. 0	8. 41
Proline	6. 67	Valine	11. 1	12. 8
Glycine	2. 11	Histidine	20. 8	21. 4
		Aspartic acid	49. 8	22. 0
		Tryptophan	4. 80	5. 25
		Methionine	5. 8	15. 2
		Tyrosine	2. 72	—

Discussion

In comparing the normal to the pathologic tissue, aspartic acid, histidine, and methionine show the greatest decreases. The significance of these differences in selecting compounds for chemotherapy is suggested by the work of Lagerkvist, Reichard, and Ehrensvärd (*13*), in which it was found that the carbon skeleton of isotopically labeled aspartic acid was incorporated by rat liver tissue into pyrimidine. Woods *et al.* (*14*) and Wahba and Shive (*15*) also showed that aspartic acid was necessary for pyrimidine and purine synthesis by *Lactobacillus arabinosus* 17-5. It is therefore reasonable to assume that analogues of aspartic acid or intermediates for which it is a precursor could interfere with the synthesis of nucleoproteins, since pyrimidines and purines are integral parts of these vital cell components. Furthermore, it is an amino acid known to participate actively in transamination reactions leading to the synthesis of other amino acids. McClure, Neuman, and McCoy (*16*) found that aspartic acid was one of the precursors of lysine.

In the second experiment analyses were made for seven amino acids in seven normal tissues from a series of normal and tumor-bearing mice. These results are presented in table 4 with further comparisons in table 3, last column. The first experiment has shown that these seven amino acids were in greater concentration in the liver than in the tumor tissue. To determine whether the amino acid composition of normal liver tissue was similar to that of other body tissues and whether it was valid to choose liver as the tissue for comparison, the following tissues from normal and tumor-bearing mice were analyzed: Kidney, liver, brain, muscle, spleen, intestine, and heart tissue from normal mice, and these same tissue types plus the tumor from tumor-bearing mice were analyzed for aspartic acid, leucine, isoleucine, valine, histidine, tryptophan, and methionine. The tissues were treated as previously described. These tissues were taken from mice with advanced tumors, *i.e.*, 13 days from implantation. The data of table 3, last column, are from this series of mice, and in general they confirm the results of the first experiment. For methionine a greater difference was found in the second experiment.

TABLE 4.—*The content of seven amino acids in seven nontumorous tissues of normal and tumor-bearing mice*

(gm. amino acid per 100 gm. lipide-free dry tissue; pooled tissues of 10 animals in each group)

Tissue	Aspartic acid	Leucine	Isoleucine	Valine	Histidine	Tryptophan	Methionine
			NORMAL MICE				
Kidney	9. 75 ± . 25	8. 12 ± . 38	4. 02 ± . 10	4. 35 ± . 09	1. 65 ± . 25	0. 635 ± . 04	1. 70 ± . 002
Liver	9. 47 ± . 13	—	3. 22 ± . 19	3. 35 ± . 09	1. 60 ± . 185	0. 706 ± . 013	1. 71 ± . 101
Spleen	7. 31 ±0. 00	7. 56 ±1. 12	3. 02 ± . 10	4. 45 ± . 25	2. 84 ± . 046	0. 491 ± . 012	1. 69 ± . 157
Brain	7. 81 ± . 54	6. 88 ± . 33	3. 82 ± . 21	4. 07 ± . 11	2. 13 ± . 065	0. 640 ± . 033	1. 71 ± . 138
Intestine	6. 12 ± . 34	7. 15 ± . 83	3. 93 ± . 38	3. 83 ± . 18	1. 59 ± . 010	0. 565 ± . 014	1. 61 ± . 081
Muscle	8. 26 ± . 73	7. 23 ± . 26	4. 31 ± . 13	4. 44 ± . 18	1. 88 ± . 157	0. 583 ± . 011	2. 27 ± . 00
Heart	8. 83 ± . 27	9. 67 ± . 43	3. 83 ± . 14	5. 45 ± . 30	3. 27 ± . 057	0. 644 ± . 025	2. 00 ± . 150
Average	8. 22	7. 76	3. 72	4. 27	2. 14	. 609	1. 81
			TUMOROUS MICE				
Kidney	13. 30 ± . 26	7. 33 ± . 43	3. 72 ± . 16	4. 30 ± . 03	1. 76 ± . 14	0. 637 ± . 048	2. 36 ± . 09
Liver	12. 45 ± . 60	6. 90 ± . 34	4. 04 ± . 02	4. 47 ± . 053	1. 78 ± . 025	0. 604 ± . 009	2. 63 ± . 02
Spleen	13. 10 ± . 99	6. 67 ± . 25	3. 25 ± . 24	4. 00 ± . 09	2. 13 ± . 066	0. 633 ± . 019	2. 01 ± . 07
Brain	15. 83 ± . 94	6. 83 ± . 28	3. 90 ± . 19	4. 06 ± . 066	1. 96 ± . 131	0. 654 ± . 031	2. 55 ± . 02
Intestine	12. 73 ±1. 32	6. 68 ± . 29	3. 39 ± . 18	3. 78 ± . 034	1. 30 ± . 070	0. 637 ± . 037	2. 47 ± . 11
Muscle	13. 86 ± . 37	6. 62 ± . 075	4. 11 ± . 17	4. 11 ± . 131	1. 64 ± . 090	0. 571 ± . 025	2. 72 ± . 02
Heart	13. 30 ± . 26	8. 33 ± . 24	3. 81 ± . 08	4. 28 ± . 2	1. 85 ± . 090	0. 636 ± . 035	2. 54 ± . 12
Average	13. 51	7. 05	3. 74	4. 14	1. 77	0. 624	2. 47
Tumor	9. 70 ± . 30	6. 12 ± . 24	3. 70 ± . 09	3. 61 ± . 16	1. 39 ± . 06	0. 622 ± . 026	2. 34 ± . 07
Percentage less in tumor*	28. 3	13. 2	—	12. 8	21. 4	—	5. 26

*Deficiencies in amino acid content of tumor tissues compared with normal tissues expressed in percentages. Values calculated from two preceding rows of this table.

Table 4 shows the results obtained with the seven normal tissues and the tumors in the second series of mice. Of particular interest are the values for aspartic acid, histidine, valine, methionine, and leucine. Comparing the aspartic acid values for normal livers and livers from tumor-bearing animals, in the first set of animals, table 2, they are 15.87 and 18.43 percent (liver from animals with 13-day tumors), respectively, and in the second set of animals, table 4, 9.47 and 12.45 percent, respectively. This represents a 16 percent increase in aspartic acid content in the liver of the tumor-bearing animals over the liver from normal animals in the first experiment and a 32 percent increase in the second. The aspartic acid content in the liver and tumor tissue of the tumor-bearing animals was 18.43 and 9.26 percent in the first experiment (table 2) and 12.45 and 9.70 percent, respectively, in the second (table 4)—a decrease of 49.5 and 22 percent.

It is noteworthy that the aspartic acid content of all of the tissues analyzed from the normal mice was considerably lower (average 8.22 %) than for the same tissues (except the tumor) from the tumorous series of animals (average 13.51%).

With respect to the concentration of the other six amino acids, there were no outstanding differences between the tissues of normal and tumorous animals, except for histidine and methionine. For histidine, the difference results from the higher values obtained in the brain, spleen, and muscle tissues of the normal mice, and for methionine, it results from generally higher values in the tumorous mice, except for muscle and heart tissue.

The last figures at the bottom of table 4 show, in percent, how much less of each amino acid the tumor contains than is in the average composition of the seven non-tumor tissues. Except for isoleucine and tryptophan, which show similar values, the differences range from 5.26 percent less for methionine to 28.3 percent less for aspartic acid.

The data indicate aspartic acid to be lower in tumor tissue than in liver and in other tissues from tumorous animals. In the second series of normal animals the average content of aspartic acid in seven tissues was lower (39%) than the average of comparable tissues from tumorous animals. Since aspartic acid is a focal point for the synthesis of other amino acids and perhaps nucleic acids, this difference may reflect an attempt in the tissues of the tumor-bearing animals to keep pace with the amino acid requirements of the rapidly growing tumor tissue. Aspartic acid must be either utilized at a more rapid rate or synthesized at a slower rate by the tumor than other amino acids, a point which may make it susceptible to attack in studies on chemotherapy of sarcoma 180 in mice.

Because of the foregoing facts further studies at present are being concentrated on the aspartic acid differences. Differences in histidine and methionine may be equally significant as a rational basis for seeking chemotherapeutic agents, but the information concerning their role in nucleic acid synthesis is not so extensive as that concerning aspartic acid.

Arginine was higher in the liver of starved animals than in normal liver as were methionine, leucine, lysine, proline, and glycine. Arginine, glutamic acid, proline, and glycine were higher in the 13-day tumor than in the liver from tumor-bearing animals. These differences were not great in any case. It would be of particular interest to know the relative activities in the liver and tumor of the systems involved in the synthesis of those amino acids where the largest differences were found. This is a point under study at present.

Summary

The amino acid composition, for 17 amino acids, of normal mouse liver, starved-mouse liver, mouse tumor tissue (sarcoma 180), and liver tissue from tumor-bearing animals has been determined by microbiological-assay procedures.

In a second series of animals seven different tissues from both normal and tumor-bearing animals were analyzed for seven amino acids.

Aspartic acid showed rather large differences between tumor and non-tumor tissue. The aspartic acid content of tumor tissue was approximately one half that of the liver from the tumor-bearing animals.

Other amino acids which showed differences of a lesser degree were valine, histidine, leucine, isoleucine, and methionine.

The possible significance of these biochemical differences in relation to a basis for seeking chemotherapeutic agents is discussed.

References

(*1*) Sauberlich, H. E., and Bauman, C. A.: The amino acid content of certain normal and neoplastic tissues. Cancer Res. 11: 67–71, 1951.

(*2*) Sassenrath, E. N., and Greenberg, D. M.: Tumor host relationships. I. Effects on free amino acid concentrations of certain tissues. Cancer Res. 14: 563–569, 1954.

(*3*) Roberts, E., and Li, Chao-T'E: Amino acids in hydrolysates of the mitochondrial fraction in normal and neoplastic tissues as studied by paper chromatography. (Abstract.) Cancer Res. 9: 613, 1949.

(*4*) Roberts, E., and Frankel, S.: Free amino acids in normal and neoplastic tissues of mice as studied by paper chromatography. Cancer Res. 9: 645–648, 1949.

(*5*) ———: Further studies on free amino acids in normal and neoplastic tissues. (Abstract.) Cancer Res. 10: 237, 1950.

(*6*) Kit, S., and Awapara, J.: Free amino acid content and transaminase activity of lymphatic tissues and lymphosarcomas. Cancer Res. 13: 694–698, 1953.

(*7*) Kögl, F., and Erxleben, H.: Zur Ätiologie der malignen Tumoren. I. Mitteilung über die Chemie der Tumoren. Ztschr. f. physiol. Chem. 258: 57–95, 1939.

(*8*) Dunn, M. S., Feaver, E. R., and Murphy, E. A.: The amino acid composition of a fibrosarcoma and its normal homologous tissue in the rat. Cancer Res. 9: 306–313, 1949.

(*9*) Shapiro, D. M., Shils, M. E., and Dietrich, L. S.: Quantitative biochemical differences between tumor and host as a basis for cancer chemotherapy. I. Vitamin B_6. Cancer Res. 13: 703–708, 1953.

(*10*) Stokes, J. L., Gunness, M., Dwyer, I. M., and Caswell, M. C.: Microbiological methods for the determination of amino acids. II. A uniform assay for the ten essential amino acids. J. Biol. Chem. 160: 35–49, 1945.

(*11*) HAC, L. R., SNELL, E. E., and WILLIAMS, R. J.: The microbiological determination of amino acids. II. Assay and utilization of glutamic acid and glutamine by *Lactobacillus arabinosus*. J. Biol. Chem. 159: 273–289, 1945.

(*12*) BARTON-Wright, E. C.: The Microbiological Assay of the Vitamin B-Complex and Amino Acids. New York, Pitman Pub. Corp., 1952.

(*13*) LAGERKVIST, U., REICHARD, P., and EHRENSVÄRD, G.: Aspartic acid as a precursor for ribonucleic acid pyrimidines. Acta chem. scandinav. 5: 1212, 1951.

(*14*) WOODS, L., RAVEL, J. M., and SHIVE, W.: Relationship of aspartic acid to pyrimidine biosynthesis. J. Biol. Chem. 209: 559–567, 1954.

(*15*) WAHBA, A. J., and SHIVE, W.: A role of aspartic acid in purine biosynthesis. J. Biol. Chem. 211: 155–161, 1954.

(*16*) MCCLURE, L. E., NEUMAN, R. E., and MCCOY, T. A.: Amino acid metabolic studies. VI. Aspartic acid-lysine interrelations in *Streptococcus faecalis* (6057). Arch. Biochem. 53: 50–55, 1954.

Recorded and Expected Mortality Among the Navajo, with Special Reference to Cancer [1]

ROBERT L. SMITH, *M.D.*,[2] CLARENCE G. SALSBURY, *M.D.*,[3] *and* ALEXANDER G. GILLIAM, *M.D.*,[2,4]
National Cancer Institute, Bethesda, Maryland, and Arizona State Department of Health, Phoenix, Arizona

During 23 years (1929–51) of practice at the Sage Memorial Hospital at Ganado, Arizona, Salsbury observed 66 cases of cancer in Navajo Indians among approximately 34,500 admissions. It is estimated that about 17,000 of these admissions represented adults and that 95 percent were Navajos. The Navajo patients observed with cancer comprise about 0.4 percent of the estimated adult admissions. Salsbury encountered no Navajo females with cancer of the breast during this period. In an earlier report on a smaller experience he (*1*) diagnosed inoperable carcinoma of the breast in one Navajo male.

The present study was undertaken as part of an effort to evaluate, from existing records, Salsbury's impression that the incidence of all cancer is low among Navajos, and that cancer of the breast rarely, if ever, occurs.

The Navajo Population

The Navajo comprise the largest single tribe of American Indians. Geographically, their Reservation consists of portions of Arizona, New Mexico, and Utah. Of the 54,320 Navajos living within the Reservation as of April 1, 1950, about 60 percent reside in Arizona, 37 percent in New Mexico, and 3 percent in Utah. An unknown number live on its fringes in Arizona, as for example around Flagstaff, and an additional 9,000 to 10,000 Navajos reside on public domain in New Mexico. In addition, varying numbers of adults leave the Reservation with their families for irregular intervals to work as migrant laborers for the railroad, seasonal agricultural workers, or miners.

The Navajo Agency of the Bureau of Indian Affairs maintains headquarters for its activities at Window Rock, New Mexico. An attempt

[1] Received for publication February 23, 1956.

[2] National Cancer Institute, National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare, Bethesda, Md.

[3] Arizona State Department of Health, Phoenix, Ariz.

[4] We wish to thank J. N. Hadley and Francis Felsman, of the Division of Indian Health, Bureau of Medical Services, U. S. Public Health Service, for their aid in furnishing data and other invaluable assistance as well as the personnel of the Navajo Agency, Window Rock, Ariz., who made it possible for us to copy death certificates filed there. Special thanks for this are due Dr. Toyo Shimizu, Acting Area Health Officer, Mr. Edward Tolson, Hospital Consultant, Miss Blakesley, Statistician, and Wilbur Morgan, Chief, Census Branch.

is made at the Agency to maintain a current roster of all Navajos. This involves a so-called "census roll," maintained by recording all known births and deaths, and supplemented by information on individuals and families obtained as a by-product of a variety of activities within the Agency. Although every effort is made to keep careful records on population and deaths, they are not believed to be entirely accurate. Too many vital-statistics jurisdictions are involved to be certain that copies of death certificates for all Navajo deceased are submitted to Window Rock, and there is no practical way to measure periodic migration accurately.

At the 1950 Census 64,374 Indians, including residents on public domain, were enumerated as residing in the Navajo Indian Agency Area. Of these, 32,444 (50.4%) were males and 31,930 (49.6%) females. Although a relatively small but unknown number of them were not Navajos, this enumeration is here considered to be the Census estimate of Navajos. A special tabulation was also made by the Bureau of the Census for the Health Branch of the Bureau of Indian Affairs, the purpose of which was to obtain special information on demographic characteristics more representative of the Navajo tribe. These include Navajos residing on the Hopi Reservation. Tribal affiliation was not recorded in this Census for 2,839 Indians living on the Reservation in New Mexico, nor for 1,774 in Arizona and 8 in Utah. These are counted as Navajos in what is here called the Special Census Study estimate. The total arrived at for the Special Census Study was 54,320, of which 27,084 (49.9%) were males and 27,236 (50.1%) females.[5]

The Navajo Agency, on the basis of data from a variety of sources, including estimates of numbers of migratory laborers and their families, estimated that the population from which death-certificate data are available was 70,567. This total is here called the Agency estimate.

The age and sex distribution of the published Census data and the Special Census Study estimates are known, but the numbers in these components have not been tabulated for the Agency estimate. In table 1 is given the percentage distribution, by age, of the Special Census Study estimate of the male and female Indians on the Navajo Reservation, which, though differing in some detail, is in general similar to the published Census data of these distributions for all Indian residents on the Navajo Reservation. That for the white and nonwhite United States population is shown for comparison. Because of the excessive proportion of Navajos in the younger ages it is clear that fewer cases of cancer would be expected among them than in a white or nonwhite population of comparable total size.

Practically all Indians both on and off the Reservation are entitled to free medical care in hospitals and clinics maintained by the Federal Government. In addition to these hospitals there are several other medical facilities supported by missions, the largest of which is the Sage

[5] These numbers were based on preliminary figures. For a more recent estimate of population, which differs slightly from the ones employed here, see Hadley (*2*).

TABLE 1.—*Percentage distribution, by age, of the male and female Navajo population, as determined in a Special Census Study, compared with the entire white and nonwhite United States population, as estimated on July 1, 1950*

Age	Navajo		Entire United States			
			Nonwhite*		White	
	Male	Female	Male	Female	Male	Female
0–14	47.6	46.0	32.5	31.1	26.9	25.7
15–24	19.4	21.0	16.1	16.8	14.6	14.4
25–34	10.8	11.8	15.1	16.2	15.6	16.0
35–44	7.9	8.5	13.7	14.2	14.2	14.3
45–54	6.1	5.6	10.6	10.2	11.6	11.6
55–64	4.0	3.3	6.7	6.3	9.1	9.1
65 and over	4.2	3.8	5.3	5.2	8.0	8.9
Total	100.0	100.0	100.0	100.0	100.0	100.0

*Of the total United States nonwhite population 95.3 percent is classed as Negro.

Memorial Hospital (Presbyterian) at Ganado. During the period of the study, it is estimated that better than 95 percent of all Navajos who received modern medical care attended one or more of the hospitals located on or contiguous to the Reservation.

In addition to the care received from modern medicine, however, an unknown amount is accounted for by the Medicine Man. No reliable information is available on the characteristics of Navajos who do and who do not utilize the facilities of modern medicine or on whether there is a tendency toward selection of one or the other type of medical care for specific types of illness. Since the Navajo are seminomadic and tend to live in small, isolated, family groups rather than in communities, and because of a variety of tribal customs, a sampling survey to acquire accurate information on this would impose serious technical and practical problems.

Methods of Study

To determine whether a deficit of cancer actually exists it is necessary to take account of the varying estimates of the number of Navajos at risk of dying as well as the fact that a high proportion of them are in the young ages of reduced cancer risk (table 1). To accomplish this, three alternative statistical procedures are available. The most generally satisfactory of the three methods, involving direct comparison of age-specific death rates, is unsuitable because of the irregular and unstable rates which result when the relatively few Navajo cancer deaths are distributed by site, age, and sex. Comparison of the Navajo risk with that in other population groups by means of total rates adjusted for age by the direct method is similarly unsuitable, and for the same reason, since the direct method requires multiplication of the unstable Navajo age-, sex-, and site-specific rates by some standard population. The only alternative, therefore, is the indirect method of age adjustment, which involves applying the more stable rates of a larger population group to the

Navajo population at risk. This procedure asks the question, how many deaths would be expected among the Navajo if the age-, sex-, and site-specific rates of some other large group of people prevailed? The deaths expected may then be compared with those actually observed.

Since it is not generally recognized, it should be pointed out that comparisons made by the indirect method do not always agree with those made by the direct method of age adjustment. The differences in results with the two methods are accentuated when diseases with strikingly different age selections are grouped, or when the populations compared are strikingly different in their distribution by age. Thus, in these data there is fairly close agreement in results obtained by the direct and indirect methods when the Navajo experience is compared with that of the nonwhite, for individual diseases; but the agreement is less marked when it is compared with the white experience. This is due to the fact, as noted in table 1, that the age distribution of the Navajo population is more similar to that of the nonwhite than the white population.

The results of comparisons of the two methods of adjustment which have been made with these data are not illustrated because of their complex nature. It may be stated, however, that disagreement is largely one of degree rather than of direction *except* when comparisons are made of deaths from all causes. In the interest of relative simplicity of presentation, this discussion will therefore be limited to comparisons made with the indirect method of adjustment.

Copies were made of all death certificates pertaining to Navajos, available at Window Rock, for the years 1948–52 inclusive. In addition to cause of death, which was coded according to the Sixth Revision of the International List, information was also obtained as to whether the death occurred in a hospital, and, if not, whether a physician was in attendance at death. The fact that the deceased was an Indian is generally adequately recorded on death certificates. The tribe of which he was a member is not regularly noted. For the Navajo specifically, however, and in all Indian Bureau facilities generally, special effort is made to have tribal affiliation recorded. While every effort is made to have copies of certificates pertaining to the Navajo submitted to the Agency at Window Rock, coverage is probably not complete for Navajos who die some distance from the Reservation and outside of Indian Bureau facilities.

Because of the uncertainty of the exact population base from which death-certificate data are available, it was deemed desirable to make an estimate of the range of deaths expected—that is, the "minimum" and "maximum" numbers expected insofar as these numbers are influenced by estimates of the number, age, and sex composition of the Navajo population at risk. Therefore, for comparison of mortality among the Navajo with that of the United States white and nonwhite populations, expected deaths among the Navajo were computed from three different population bases: 1) the published Census enumeration (64,374); 2) the Agency total (70,567) distributed by age and sex in the same proportion as the Census enumeration; and 3) the Agency total distributed by age

and sex as in the Special Census Study estimate. These three population estimates (by age and sex) were multiplied by the age-, sex-, and cause-specific 1950 death rates for the entire white and for the entire nonwhite United States population. The results, multiplied by 5, therefore comprise the number of deaths from each cause expected to occur among the Navajo in the 5 years centering around 1950, if they had been subjected to the same age- and sex-specific rates prevailing in the United States white or nonwhite population as a whole. As might be anticipated, computations employing the published Census enumeration estimates uniformly yielded the lowest number of deaths expected. The largest number of deaths expected was derived when use was made of the Agency estimate. For males this estimate distributed as the Census enumeration always yielded the maximum figure, while for females there were only minor differences between deaths expected using the same estimate and the Agency estimate distributed as the Special Census Study.

Deaths observed for any cause, divided by the deaths expected (as derived by the procedures outlined above) yield a ratio. This ratio multiplied by 100 gives a Standardized Mortality Ratio (SMR), which numerically represents the percentage of the United States average annual death rate constituted by the Navajo rate, adjusted for age differences by the indirect method.

In addition to computing mortality ratios for cancer, such ratios have also been obtained for deaths due to all causes as well as for deaths attributed to diseases grouped in conventional broad causes. The purpose of these computations is to determine what takes the place of cancer as a stated cause of death in the event a deficit of cancer appears to exist.

General Results

Table 2 shows the number of deaths recorded in 5 years among Navajo males, by broad causes of death. It also shows the minimum and maximum numbers, as previously defined, which would have been expected if the risk of dying from these causes had been similar to that prevailing for the general United States white and nonwhite populations. The resultant standardized mortality ratios (SMR) are also given where the numbers of deaths expected or observed are numerically sufficient for reasonably stable ratios.

When broad causes of death are considered for males (table 2), it is observed that all estimates yield excessive mortality from the following classes of disease: (I) infective and parasitic, (VIII) respiratory, (IX) digestive diseases (largely gastroenteritis and colitis), (XVI) senility and other ill-defined causes, and (XVII) violence. In addition, diseases of early infancy (XV) are materially greater than would be expected from experience of the white population. Substantial deficits are noted for (II) neoplastic diseases, (VI) diseases of the central nervous system, and (VII) circulatory diseases, when compared with both white and nonwhite experience. Diseases of the genitourinary system (X) are relatively infrequent only when compared with white experience. For the other

TABLE 2.—*Deaths recorded among Navajo* males *during 1948–52, by broad causes; deaths expected on the basis of age-specific rates observed in 1950 among U. S. white and nonwhite males; and resultant standardized mortality ratios (SMR)*

Broad cause of death	International list number	Number of deaths recorded	On basis of nonwhite rates				On basis of white rates			
			Number of deaths expected		SMR		Number of deaths expected		SMR	
			Minimum*	Maximum*	Minimum	Maximum	Minimum*	Maximum*	Minimum	Maximum
I Infective and parasitic	(001–138)	399	139	153	261	287	42	46	867	950
II Neoplasm	(140–239)	43	126	139	31	34	137	152	28	31
Malignant	(140–205)	36	123	135	27	29	134	148	24	27
Benign	(210–239)	7	3	4	†	†	3	4	†	†
III Allergy, endocrine, etc	(240–289)	10	21	23	43	48‡	18	20	50‡	56‡
IV Blood-forming organs	(290–299)	4	5	6	†	†	4	4	†	†
V Mental diseases	(300–326)	10	7	8	†	†	4	4	†	†
VI Nervous and sensory	(330–398)	64	164	180	36	39	107	118	54	60
VII Circulatory	(400–468)	73	449	493	15	16	425	467	16	17
VIII Respiratory	(470–527)	251	120	132	190	209	55	60	418	456
IX Digestive	(530–587)	305	67	73	418	455	45	50	610	678
X Genitourinary	(590–637)	29	69	76	38	42	32	35	83‡	91‡
XI Pregnancy and puerperium	(640–689)	—	—	—	—	—	—	—	—	—
XII Skin and cellular tissue	(690–716)	8	2	2	†	†	1	1	†	†
XIII Bones and organs of movement	(720–749)	1	2	3	†	†	2	2	†	†
XIV Congenital malformation	(750–759)	31	26	29	107‡	119‡	32	35	89‡	97‡
XV Early infancy	(760–776)	166	179	197	84‡	93‡	112	122	136	148
XVI Senility and ill-defined	(780–795)	203	90	98	207	226	15	16	1, 269	1, 353
XVII Violence	(800–999)	267	215	236	113‡	124	134	147	182	199
All causes	.....	1, 864	1, 681	1, 848	101‡	111	1, 165	1, 279	146	160

*The minimum and maximum numbers of deaths expected are dependent upon different estimates of the number and age composition of the Navajo population at risk.

†Numbers of expected or observed deaths are too few for computation of reliable SMR's.

‡Not significantly different from 100. All other SMR's are significantly different from 100 at a probability level of $P=0.01$ or less.

broad causes, deaths expected are too few for much significance to be attached to the results.

The general features of expected and observed mortality among females are similar to that of the males, as is evident in table 3, except that deaths due to violence (XVII) do not appear to be excessive. Also noteworthy is the excess Navajo mortality for diseases of pregnancy and the puerperium, when compared to white rates.

The ratios computed for all causes of death combined must be accepted with reservations since, as previously pointed out, neither the direct nor indirect methods of adjustment are satisfactory when diseases of widely different age selection are grouped together. The only satisfactory comparison between the Navajo and the white and nonwhite experience, for all causes of death combined, is obtained by direct comparison of age-specific rates. This procedure is acceptable since all deaths for a 5-year period are sufficient to produce reasonably stable rates. Since such comparisons have been made previously (*2*) they will not be repeated here.

Observed and Expected Mortality Attributed to Cancer

For males, mortality attributed to cancer is estimated to be from 24 to 29 percent of that expected, and among females from 31 to 36 percent (tables 2 and 3). When the observed cancer deaths are considered by site of cancer, their distribution suggests that the Navajo might experience a somewhat different predilection for cancer of specific sites than either the white or nonwhite population. Expected deaths for a number of specific sites were therefore computed and the results of these computations, together with recorded deaths, are given in table 4. As would be anticipated the numbers are so small, even with a 5-year experience, that little significance can be attached to the results except for a few specific cancers.

Cancer of the digestive tract as a whole appears to be recorded less commonly than expected among both males and females. This deficit tends to involve all portions of the gastrointestinal tract, except for cancer of the pancreas in the male and primary cancer of the liver in the female. In each instance the numbers are too small to be of much significance, but it should be noted that a numerical excess of cancer of the liver is recorded in females.

There is a clear deficit of cancer of the lung in the male. While no deaths attributed to respiratory cancer were recorded among females, the numbers expected are quite small.

Among males there appears to be a deficit of cancer of the genitourinary system. This is marked for cancer of the prostate, the only single site which contributed much to the total expected. In the females, there is a clear deficit of cancer of the breast, with 1 death recorded and from 13 to 20 expected. Cancer of the cervix, on the other hand, was recorded about as expected on the basis of white experience but less commonly than expected had nonwhite rates prevailed.

‡Not significantly different from 100. All other SMR's are significantly different from 100 at a probability level of $P=0.01$ or less.

TABLE 3.—*Deaths recorded among Navajo females during 1948–52, by broad causes; deaths expected on the basis of age-specific rates observed in 1950 among U. S. white and nonwhite females; and resultant standardized mortality ratios (SMR)*

Broad cause of death	International list number	Number of deaths recorded	On basis of nonwhite rates				On basis of white rates			
			Number of deaths expected		SMR		Number of deaths expected		SMR	
			Minimum*	Maximum*	Minimum	Maximum	Minimum*	Maximum*	Minimum	Maximum
I Infective and parasitic	(001–138)	473	97	106	446	488	23	26	1, 819	2, 057
II Neoplasm	(140–239)	41	118	130	32	35	109	120	34	38
Malignant	(140–205)	38	110	121	31	35	105	116	33	36
Benign	(210–239)	3	8	9	†	†	4	4	†	†
III Allergy, endocrine, etc	(240–289)	8	27	30	27	30	20	22	36	40
IV Blood-forming organs	(290–299)	3	5	5	†	†	3	4	†	†
V Mental diseases	(300–326)	2	3	4	†	†	2	2	†	†
VI Nervous and sensory	(330–398)	56	145	159	35	39	85	93	60	66
VII Circulatory	(400–468)	54	333	365	15	16	238	261	21	23
VIII Respiratory	(470–527)	227	91	100	227	249	36	39	582	631
IX Digestive	(530–587)	239	45	50	478	531	28	31	771	854
X Genitourinary	(590–637)	17	46	50	34	37	16	18	94‡	106‡
XI Pregnancy and puerperium	(640–689)	29	19	20	145‡	153‡	4	4	725	725
XII Skin and cellular tissue	(690–716)	3	2	2	†	†	1	1	†	†
XIII Bones and organs of movement	(720–749)	1	3	3	†	†	2	2	†	†
XIV Congenital malformation	(750–759)	19	21	23	83‡	90‡	27	30	63‡	70‡
XV Early infancy	(760–776)	136	137	151	90‡	99‡	77	85	160	177
XVI Senility and ill-defined	(780–795)	198	66	73	271	300	9	10	1, 980	2, 200
XVII Violence	(800–999)	74	80	88	84‡	92‡	51	56	132‡	145
All causes	.....	1, 580	1, 238	1, 359	116	128	731	804	197	216

*The minimum and maximum numbers of deaths expected are dependent upon different estimates of the number and age composition of the Navajo population at risk.

†Number of expected or observed deaths are too few for computation of reliable SMR's.

‡Not significantly different from 100. All other SMR's are significantly different from 100 at a probability level of $P=0.01$ or less.

For both males and females, cancers of the brain and of the bone were about as common as expected. The deficit in leukemia and the lymphomas appears to be less pronounced among the males than the females.

Discussion

In evaluating these comparisons of expected and observed causes of death, it must be borne in mind that the SMR's calculated for deaths attributed to senility and ill-defined causes varied from 207 to 1,353 among the males and from 271 to 2,200 in the females (tables 2 and 3). In addition, a substantial number of the deceased were unattended by a physician at death. Table 5 shows the deaths, distributed by age and sex, which were unattended by a physician and those which were attributed to senility and other ill-defined causes. Since deaths due to violent causes should, in general, be readily classified without much medical observation, percentages based on all deaths except those attributed to violence are also given in table 5.

It is observed that the proportion unattended by a physician increased with age and comprised nearly 40 percent of the 522 deaths (exclusive of violent deaths) which occurred in the ages of greatest cancer risk, that is, in persons 45 years of age and older. This does not necessarily mean, however, that the assigned cause of death in all of these was unsupported by medical observation. In some, but unfortunately in an unknown number and proportion of instances, the cause of illness had been established in a hospital, but because of tribal customs the patient had left the hospital to die unattended by the physician signing the certificate. It was established, for example, that the unattended death attributed to cancer of the breast (table 4) had previously been so diagnosed in a hospital. However, because of the tendency of the individual Navajo to use a number of different names, it was not possible to make a systematic check of the frequency, in those unattended at death, with which the certified cause of death was based on reliable medical information.

Table 5 also shows that deaths attributed to senility and other ill-defined causes increased with age and comprised 138 (26 percent) of the 522 deaths (exclusive of violent deaths) recorded in those 45 years of age and older. If most of these deaths had actually been due to cancer they would have been sufficient to account for the maximum cancer deficit computed for these ages. However, the deaths which were either attributed to senility or were unattended by a physician (comprising 215 of the 522 deaths in those 45 and over) taken all together are insufficient to account for the combined deficit in deaths due to cancer, cardiovascular diseases, and diseases of the central nervous system. It would be expected that these causes would share proportionately if the correct cause of death could be assigned.

Unpublished data, show that Navajo age-specific death rates for all causes are substantially less among the older Navajos than recorded for all American Indians. While this fact suggests that the recording system was less efficient in identifying as Navajos some Indians dying in the older

TABLE 4.—*Deaths attributed to cancer among the Navajo during 5 years, 1948–52, and deaths expected among them on the basis of age- and sex-specific rates observed in 1950 among the white and nonwhite U. S. populations*

Cancer site	International list number	Number of male deaths recorded	Expected deaths among males on basis of—				Number of female deaths recorded	Expected deaths among females on basis of—			
			White rates		Nonwhite rates			White rates		Nonwhite rates	
			Min.*	Max.*	Min.*	Max.*		Min.*	Max.*	Min.*	Max.*
All sites	(140–205)	36	134. 5	147. 7	122. 7	134. 7	38	105. 2	115. 6	109. 8	120. 7
Buccal cavity and pharynx	*(140–148)*	*0*	*5. 0*	*5. 5*	*3. 8*	*4. 2*	*0*	*1. 0*	*1. 1*	*1. 3*	*1. 5*
Digestive tract	*(150-156A, 157-159)*	*14*	*53. 5*	*58. 8*	*55. 9*	*61. 3*	*19*	*35. 3*	*38. 8*	*32. 5*	*35. 6*
Esophagus	(150)	0	3. 3	3. 6	5. 8	6. 4	1	0. 7	0. 8	1. 4	1. 5
Stomach	(151)	5	17. 7	19. 5	24. 1	26. 5	4	8. 4	9. 3	10. 7	11. 8
Intestines	(152–153)	2	12. 9	14. 2	8. 1	8. 8	3	12. 5	13. 7	8. 5	9. 3
Rectum	(154)	2	7. 3	8. 0	5. 6	6. 2	0	4. 2	4. 6	4. 0	4. 4
Liver and biliary passages, primary	(155)	0	2. 2	2. 4	2. 2	2. 4	5	2. 7	3. 0	1. 4	1. 5
ditto, unstated primary or secondary	(156A)	0	3. 0	3. 3	3. 9	4. 3	3	2. 4	2. 7	2. 8	3. 1
Pancreas	(157)	5	6. 2	6. 8	4. 9	5. 3	2	3. 6	3. 9	2. 6	2. 8
Peritoneum and other, and unspec.	(158–159)	0	0. 9	1. 0	1. 3	1. 4	1	0. 8	0. 8	1. 1	1. 2
Respiratory system	*(160–164)*	*2*	*19. 9*	*21. 8*	*15. 3*	*16. 8*	*0*	*3. 8*	*4. 2*	*3. 4*	*3. 7*
Trachea, bronchus, and lung	(162–163)	1	17. 1	18. 7	12. 9	14. 2	0	3. 3	3. 6	2. 8	3. 0
Other	(160, 161, 164)	1	2. 8	3. 1	2. 4	2. 6	0	0. 5	0. 6	0. 6	0. 7
Breast and genitourinary organs	*(170–181)*	*4*	*24. 4*	*26. 8*	*24. 3*	*26. 8*	*10*	*42. 9*	*47. 2*	*55. 0*	*60. 4*
Breast	(170)	0	0. 3	0. 3	0. 3	0. 4	1†	18. 5	20. 3	15. 4	16. 9
Uterus	(171–174)	–	–	–	–	–	8	14. 4	15. 9	31. 4	34. 5
Cervix	(171)	–	–	–	–	–	7	7. 6	8. 4	17. 2	18. 9
Other and unspec. uterus	(172–174)	–	–	–	–	–	1†	6. 8	7. 5	14. 2	15. 6
Other genitourinary	(175–181)	4	24. 1	26. 5	24. 0	26. 4	1	10. 0	11. 0	8. 2	9. 0
Ovary and other, and unspec. female genital	(175–176)	–	–	–	–	–	1	6. 4	7. 0	4. 7	5. 2
Prostate	(177)	2†	14. 4	15. 8	17. 3	19. 0	–	–	–	–	–

Prostate	(177)	2†	14.4	15.8	17.3	19.0	–	–	–	–	–
Testis and other, and unspec. male genital	(178–179)	2	1.4	1.6	1.0	1.1	–	–	–	–	–
Kidney, bladder, and other urinary	(180–181)	0	8.3	9.1	5.7	6.3	0	3.6	4.0	3.5	3.8
Other and unspecified sites	*(156B, 165, 190–199)*	*10*	*16.1*	*17.7*	*13.5*	*14.9*	*7*	*12.2*	*13.4*	*11.2*	*12.4*
Skin	(190–191)	0	2.7	3.0	0.9	1.0	0	1.6	1.8	0.9	1.0
Brain and other CNS	(193)	2	4.2	4.7	2.4	2.6	2	2.9	3.2	1.6	1.8
Bone and connective tissue	(196–197)	3‡	2.4	2.6	2.1	2.4	2	1.4	1.6	1.4	1.5
Other	(156B, 165, 192, 194, 195, 198–199)	5§	6.8	7.4	8.1	8.9	3†	6.3	6.8	7.3	8.1
Leukemia and lymphoma	*(200–205)*	*6*	*15.6*	*17.1*	*9.9*	*10.7*	*2*	*10.0*	*10.9*	*6.4*	*7.1*
Leukemia	(204)	3	8.9	9.8	4.9	5.3	1	6.3	6.9	3.5	3.9
Lymphoma	(200–203, 205)	3	6.7	7.3	5.0	5.4	1	3.7	4.0	2.9	3.2

*The minimum and maximum numbers of deaths expected are dependent upon different estimates of the number and age composition of the Navajo population at risk.

†One case, death certificate signed by physician not in attendance.

‡Two cases, death certificate signed by physician not in attendance.

§Three cases, death certificate not signed by a physician.

The remaining 65 other-cancer deaths occurred in hospitals.

TABLE 5.—*Deaths recorded among the Navajo, 1948–52. Number attributed to violent and accidental causes; number and percent unattended by a physician; and number and percent attributed to senility and other ill-defined causes*

Age at death	Number of deaths all causes			Deaths, all causes, unattended by a physician			Deaths attributed to violence			Deaths attributed to senility and other ill-defined causes		
	Male	Female	Total	Male	Female	Total	Male	Female	Total	Male	Female	Total
0–14	1, 119	984	2, 103	170	180	350	51	35	86	95	93	188
15–24	157	143	300	85	48	133	53	10	63	15	18	33
25–34	145	131	276	79	42	121	62	8	70	10	17	27
35–44	93	77	170	47	30	77	42	7	49	6	9	15
45–54	88	52	140	52	18	70	30	6	36	11	8	19
55–64	68	56	124	32	18	50	14	1	15	14	18	32
65 and over	194	137	331	83	56	139	15	7	22	52	35	87
45+	350	245	595	167	92	259	59	14	73	77	61	138
Total	1, 864	1, 580	3, 444	548	392	940	267	74	341	203	198	401

Age at death	Percentage of all deaths unattended by physician			Percentage of all deaths attributed to senility, etc.			Percentage of all deaths, except violent, unattended by physician			Percentage of all deaths, except violent, attributed to senility, etc.		
	Male	Female	Total	Male	Female	Total	Male	Female	Total	Male	Female	Total
0–14	15. 2	18. 3	16. 6	8. 5	9. 5	8. 9	13. 7	17. 2	15. 3	8. 9	9. 8	9. 3
15–24	54. 1	33. 6	44. 3	9. 6	12. 6	11. 0	39. 4	29. 3	33. 8	14. 4	13. 5	13. 9
25–34	54. 5	32. 1	43. 8	6. 9	13. 0	9. 8	31. 3	28. 5	29. 6	12. 0	13. 8	13. 1
35–44	50. 5	39. 0	45. 3	6. 5	11. 7	8. 8	29. 4	34. 3	32. 2	11. 8	12. 9	12. 4
45–54	59. 1	34. 6	50. 0	12. 5	15. 4	13. 6	43. 1	28. 3	36. 5	19. 0	17. 4	18. 3
55–64	47. 1	32. 1	40. 3	20. 6	32. 1	25. 8	46. 3	30. 9	38. 5	25. 9	32. 7	29. 4
65 and over	42. 8	40. 9	42. 0	26. 8	25. 5	26. 3	40. 2	39. 2	39. 8	29. 1	26. 9	28. 2
45+	47. 7	37. 6	43. 5	22. 0	24. 9	23. 2	41. 9	35. 1	38. 9	26. 5	26. 4	26. 4
Total	29. 4	24. 8	27. 3	10. 9	12. 5	11. 6	21. 9	22. 7	22. 3	12. 7	13. 1	12. 9

ages, it is also consistent with the idea that this tribe is subject to different forces of mortality than are operating for Indians as a whole.

Thus, though these data as they stand show a deficit for some forms of cancer among the Navajo, there is uncertainty about the true cause of death in a substantial portion of the older Navajos and some question as to accuracy of identification as Navajo among some of the elderly Indian dead.

The question may also be raised as to whether some of the very large excess Navajo mortality certified as due to diseases of the respiratory and digestive systems (tables 2 and 3) was actually due to cancer. That this is unlikely is indicated by the fact that the excess is almost entirely limited to deaths in Navajos under 5 years of age. This is also true, though to a lesser degree, for the excess attributed to respiratory tuberculosis. For this disease excess mortality occurred principally in those under 45 years of age and was only moderate for those 45 and over. The excess charged to respiratory tuberculosis in the older ages, however, is great enough to account for the deficit of cancer of the lung, if a high proportion of them had been erroneously certified.

Conclusion

The data presented are consistent with the belief that the Navajo experience a deficit of cancer as a whole and, more specifically, of cancer of the breast, prostate, and lung. This apparent deficit of cancer cannot be regarded as an established fact, however, because of the high proportion (41%) of all deaths among those 45 years of age and older that were either unattended by a physician or were attributed to senility and other ill-defined causes and because of the fact that the age-specific death rates for all causes in the older Navajo are substantially less than the corresponding rates for all American Indians. It is clear, nevertheless, that the numbers of deaths which were unreliably certified as to cause are insufficient to account for the combined deficit of cancer and diseases of the cardiovascular and the central nervous systems.

References

(*1*) SALSBURY, C. G.: Incidence of certain diseases among the Navajos. Arizona Med. 4: 29–31, 1947.

(*2*) HADLEY, J. N.: Health conditions among Navajo Indians. Pub. Health Rep. 70: 831–836, 1955.

Virus of Avian Erythroblastosis. II. Influence of Host Age and Route of Inoculation on Dose-Response [1,2]

G. S. Beaudreau, R. A. Bonar, D. Beard, *and* J. W. Beard, *Department of Surgery, Duke University School of Medicine, Durham, North Carolina*

Host response to viruses may be influenced by many factors. Frequently, the patterns of response resulting from the impression of a given influence may serve as an aid in the characterization of a particular host-virus interaction. Some of the factors known to affect host response are the route of virus inoculation and the age of the host at the time of contact with the agent. Earlier studies have shown (*1*) marked effects of these factors on the induction of leukemia in chicks by the virus of avian myeloblastosis. A similar investigation has been made with the agent of another form of avian leukemia, the virus of erythroblastosis. It has been observed that, whereas the influence of route of inoculation affects host response essentially alike in both conditions, there is, as already indicated briefly (*2*), a large difference with respect to the effect of age. In myeloblastosis, host susceptibility decreases rapidly with age (*1*), while in erythroblastosis susceptibility, as measured by time from inoculation of the virus to death of the host, appears actually to increase with age. The results of these experiments on route of inoculation and age on host response to the virus of erythroblastosis are described in the present paper.

Materials and Methods

The virus employed in these, as in the earlier experiments (*2*) with erythroblastosis, was obtained in February 1955, from Dr. Astrid Fagraeus (State Bacteriological Laboratory, Stockholm) as a strain initially derived by Dr. J. Engelbreth-Holm (Department of Pathological Anatomy, University of Copenhagen). The results of early passages of the agent in this country and the characteristics of the disease, which were practically identical with those found for erythroblastosis by Furth (*3*) and Engelbreth-Holm (*4*), have been described in a previous paper (*2*). No changes have occurred thus far in the manifestations of this leukemia.

Virus for the present studies was that in the plasma of blood drawn by heart puncture from diseased birds into heparin (*5*). After removal

[1] Received for publication February 22, 1956.

[2] This work was supported by a research grant to Duke University from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service; from the American Cancer Society, on recommendation of the Committee on Growth; and by the Dorothy Beard Research Fund.

of the cells by centrifugation, first at 1,000 × g for 10 minutes and then at 1,500 × g for 20 minutes, the latter in collodion tubes, the plasmas in all instances were filtered with celite and passed afterward through Selas filters of 02 porosity (*5*). Dilutions for preparation of the respective inoculums were made, as usual, with BSA-Simm's solution (*6*).

Donors of the plasmas were chosen on the basis of the concentration of primitive cells (erythroblasts) seen in routine blood smears taken at daily intervals. Bleeding was postponed until the birds were practically moribund or obviously within a few hours of death. It is not known whether this is the best sequence for obtaining the agent in the highest concentration. Selection of the donors could be made only in this manner, however, since the micro adenosinetriphosphatase reaction (*7*), so useful in the studies on myeloblastosis, could not be applied for preliminary estimation of virus concentration in the plasma in erythroblastosis.

Measurement of virus infectivity was made by analyses based on the length of the period in days between inoculation of the virus and the time of death of the diseased host as described in an earlier report (*2*). As in the earlier work, deaths were recorded twice daily, at 11 a.m. and 11 p.m. The analyses, however, were based, except in a few instances, on the daily count obtained at 11 p.m. The volumes of the inoculums were routinely 0.1 ml., and the test hosts were inbred White Leghorn chicks of line 15 (*8*) developed (*9,10*) at the Regional Poultry Research Laboratory, East Lansing, Michigan. In the studies on route of inoculation, the chicks were 10 days old. Injections were made, as in the case of similar experiments (*1*) on the virus of myeloblastosis: 1) intravenously in the wing vein; 2) into the medullary canal of the tibia; 3) into the muscle of the leg; 4) into the peritoneal cavity; and 5) into the subcutaneous tissue over the right breast muscle.

The study on chicks of different ages was made in a single experiment with the same inoculums. The birds, hatched at intervals of 2 weeks between July 2 and September 10, 1955, were derived from eggs of the same laying flock kept at the laboratory. All the injections were made into the wing vein.

Results

Influence of route of inoculation on host response.—The data collected in two similar experiments of this category are given in table 1. In experiment 180, intravenous inoculations were made in 4 dose groups, and 2 dose groups were employed for each of the other routes. Three dose groups were inoculated intravenously in experiment 182 and 2 by each of the other routes, as in experiment 180. Each dose group consisted initially of 30 or 31 chicks, and the corrected number of birds inoculated was derived as already described (*2*).

The results were analyzed in the usual way (*2,6*) by construction of the time-frequency distributions (accumulated positive inoculations transformed to probits and plotted against log latent period in days) of the responses in each dose group as illustrated in text-figure 1. Here are shown the distributions observed with 2 doses, $10^{-1.69}$ and $10^{-3.69}$ ml. of

TABLE 1.—*The response of 10-day-old chicks to the virus of erythroblastosis administered by different routes*

Expt. no.	Route of inoculation*	Log dose plasma (*ml.*)	Corrected no. chicks inoculated†	Median log latent period (*days*)	Median latent period (*days*)	Total positive (*percent*)
180	IV	−1. 69	30	0. 935	8. 6	100
	IMed	−1. 69	28	0. 958	9. 08	100
	IMus	−1. 69	30	1. 015	10. 3	100
	IP	−1. 69	30	1. 075	11. 9	96. 6
	SC	−1. 69	29	1. 110	12. 9	93. 1
	IV	−2. 69	30	0. 975	9. 44	100
	IV	−3. 69	30	1. 055	11. 3	86. 6
	IMed	−3. 69	26	1. 060	11. 5	100
	IMus	−3. 69	31	1. 111	12. 9	93. 6
	IP	−3. 69	27	R‡	R	55. 6
	SC	−3. 69	26	R	R	80. 8
	IV	−4. 69	29	1. 115	13. 0	100
182	IV	−1. 69	30	0. 927	8. 44	100
	IMed	−1. 69	30	0. 95	8. 9	100
	IMus	−1. 69	30	1. 022	9. 58	100
	IP	−1. 69	30	1. 097	12. 5	86. 7
	SC	−1. 69	30	1. 096	12. 5	93. 3
	IV	−2. 69	30	1. 0	10	100
	IV	−3. 69	30	1. 037	10. 9	100
	IMed	−3. 69	30	0. 982	9. 58	100
	IMus	−3. 69	30	1. 125	13. 3	80
	IP	−3. 69	30	1. 170	14. 8	70
	SC	−3. 69	30	1. 233	17. 1	60

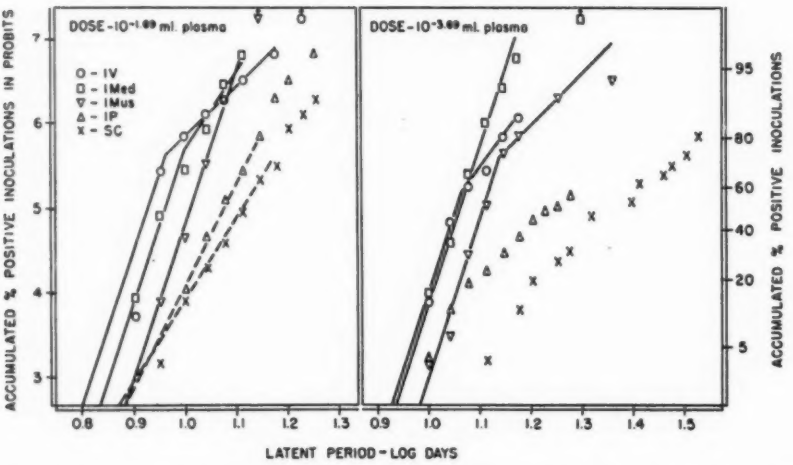
*IV=intravenous; IMed=medullary canal of tibia; IP=peritoneum; SC=subcutaneous tissue; and IMus=muscle.

†See reference (*2*).

‡R signifies abnormal patterns of response related to high levels of resistance.

plasma, given by the different routes in experiment 180. In the previous study of the virus of erythroblastosis (*2*), such time-frequency distributions were observed to be truncated, as in myeloblastosis (*6*), and the slopes of the linear distributions of the first limbs of the curves were essentially constant. Employing the value of the slope already reported (*2*), the reciprocal of the standard deviation—0.052, the lines were constructed, graphically, through the points as illustrated. The lines through the points of the truncated portions of the limbs were drawn by sight.

It is seen that with the larger dose, $10^{-1.69}$ ml. plasma, the distributions resulting from intravenous, intramedullary, and intramuscular inoculation were closely similar to those previously observed (*2*). However, a difference was seen in the results with the inoculums given intraperitoneally and subcutaneously. In these cases, the observed relations, although essentially linear in the first portions (*i.e.*, in the regions of less than about 70% positive inoculations), deviated from the common slope rather markedly for the material given subcutaneously. Truncation was slight, if it occurred at all, in these instances. Nevertheless, an estimate of host response suitable for the purposes of the experiment could be obtained by graphic construction of straight lines through the obviously linear portion of the relations as shown by the broken lines in text-figure 1.


DOSE - 10^-1.69 ml. plasma
DOSE - 10^-3.69 ml. plasma
O - IV
□ - IMed
▽ - IMus
Δ - IP
x - SC
ACCUMULATED % POSITIVE INOCULATIONS IN PROBITS
ACCUMULATED % POSITIVE INOCULATIONS
LATENT PERIOD - LOG DAYS

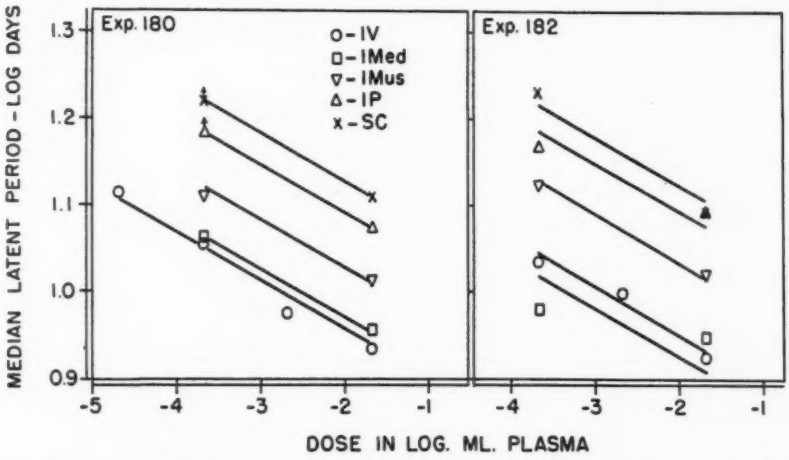
TEXT-FIGURE 1.—Time-frequency distributions of the response of 10-day-old chicks to two different doses of erythroblastosis virus, administered by different routes of inoculation in experiment 180 (table 1). The continuous lines through the points were drawn with the slope constant for this agent in line 15 chicks. The broken lines were drawn for dose $10^{-1.69}$ ml. plasma given intraperitoneally and subcutaneously as the best fit by sight. The responses to the inoculation of $10^{-3.69}$ ml. of plasma given into muscle, peritoneal cavity, and subcutaneous tissue were too low for analysis.

With the dose $10^{-3.69}$ ml. plasma, there was still greater deviation of the response to intraperitoneal and subcutaneous injections—so much so that the results were not employed for further analysis. The points observed with the respective routes of inoculation seen in text-figure 1 clearly reveal the difference between responses to the amount of virus inoculated intraperitoneally and subcutaneously and that to the same dose given by the other routes. Greater conformity was observed in experiment 182, and all the data of that experiment were applicable to further analysis.

From graphs such as those of text-figure 1 (*2*) the values of median log latent period were obtained (latent period at the level of 50% positive or probit 5). The resulting values are shown in relation to log dose, in ml., of plasma in text-figure 2, and the lines of slope, 0.054, constant for this virus in the host described (*2*), were fitted to the points by sight. The relations indicate the differences in levels of host response to the agent inoculated by the different routes, as summarized in table 2. These values were obtained by calculations of potency differences from the relations of text-figure 2 and are expressed in terms of "relative resistance" of the host, employing the response to intravenous injection as unity. Except for a single grossly aberrant point (dose-group $10^{-3.69}$ injected into the medullary canal in experiment 182), the results were uniform in revealing the extreme differences to be expected under the various conditions. As in the case of myeloblastosis, there was little difference between the intravenous and

intramedullary routes. The difference was about 10- to 20-fold with the intramuscular inoculation. It is notable that the findings with intravenous, intramedullary, and intramuscular inoculations of the agent of erythroblastosis were practically identical with those observed (*1*) with the virus of myeloblastosis. Large differences between the two diseases, however, were seen in the findings with intraperitoneal and subcutaneous injections. In these instances the apparent relative resistance of the hosts was of two and three orders of magnitude greater, respectively, than that of chicks injected intravenously. This was a considerable divergence from the observations in myeloblastosis (*1*).

Influence of age on host response.—The results of the experiment with chickens of 6 different ages, varying from 7 to 77 days, are summarized in table 3. Time-frequency distributions were constructed as for the findings with routes of inoculation. Inspection of the resulting graphs indicated, first, that the patterns of response were basically similar for



TEXT-FIGURE 2.—The relations of median log latent period in days to log dose of the virus of erythroblastosis given by various routes of inoculation in experiments 180 and 182. The points with arrows directed upwards were indeterminate because of the low responses to intraperitoneal and subcutaneous inoculations. The lines were constructed graphically with the slope constant for the relationship in erythroblastosis.

TABLE 2.—*Relative resistance of 10-day-old chicks to injection with the virus of erythroblastosis given by different routes*

Route of inoculation	Relative resistance		
	Expt. 180	Expt. 182	Average
Intravenous	1.0	1.0	1.0
Intramedullary	1.8	0.5	1.2
Intramuscular	19.8	24.5	22.1
Intraperitoneal	317	468	393
Subcutaneous	1,300	1,500	1,400

all age groups and, consequently, there was no dependence of the nature of response on age. This corroborated previous evidence of the similarity of the responses of 10- and 54-day-old chicks. It has been evident in the present work, however, that although the relations exhibit the usual truncation and uniformity or constancy of the slopes of the first limbs, the time-frequency responses of the older chicks varied somewhat more than those of the younger ones. The variation occurred principally in the region of the earliest deaths. The data showed clearly, as substantiated by the total findings, that the older chicks were more susceptible to the agent and succumbed earlier than the younger birds. It was evident, however, that despite the variation occurring in some of the dose groups of older birds, the data were entirely suitable for analysis by the usual methods.

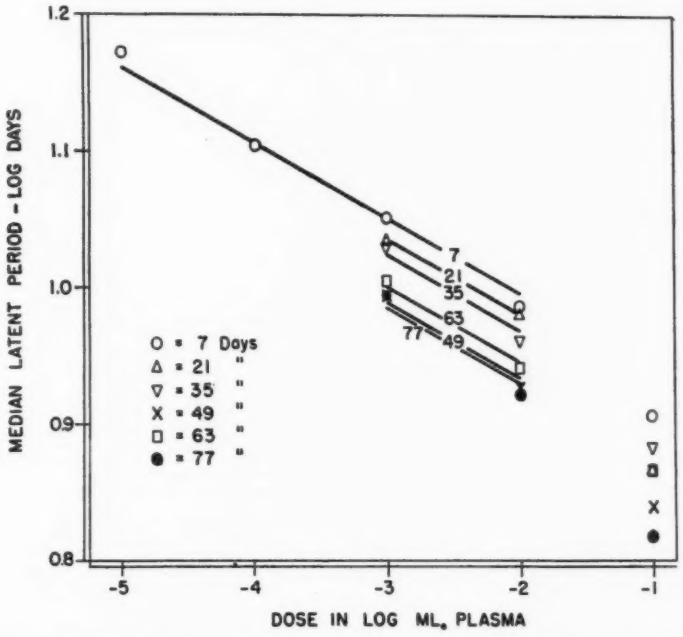
TABLE 3.—*The response of chicks of different ages to intravenous inoculation with the virus of erythroblastosis*

Age of chicks (*days*)	Log dose plasma (*ml.*)	Corrected* no. chicks inoculated	Median log latent period (*days*)	Median latent period (*days*)	Total positive (*percent*)
7	10^{-1}	30	0. 905	8. 04	96. 6
21	10^{-1}	31	0. 867	7. 3	96. 8
35	10^{-1}	28	0. 882	7. 62	89. 3
49	10^{-1}	30	0. 840	6. 92	100
63	10^{-1}	29	0. 865	7. 32	86. 2
77	10^{-1}	30	0. 817	6. 63	100
7	10^{-2}	30	0. 985	9. 66	93. 3
21	10^{-2}	29	0. 981	9. 56	96. 6
35	10^{-2}	30	0. 961	9. 14	100
49	10^{-2}	30	0. 927	8. 45	100
63	10^{-2}	30	0. 940	8. 7	90
77	10^{-2}	30	0. 922	8. 4	100
7	10^{-3}	30	1. 052	11. 3	93. 3
21	10^{-3}	30	1. 035	10. 8	96. 6
35	10^{-3}	30	1. 032	10. 8	93. 3
49	10^{-3}	30	0. 998	9. 95	96. 6
63	10^{-3}	30	1. 002	10. 3	93. 3
77	10^{-3}	30	0. 995	9. 9	96. 6
7	10^{-4}	30	1. 105	12. 7	96. 6
7	10^{-5}	29	1. 172	14. 9	79. 3

*See reference (*2*).

Further evidence of the qualitative and quantitative aspects of the responses is obtained from the relations of log latent period to log dose, in ml., of plasma as shown in the graph of text-figure 3. The individual points represent the values of median log latent period for the respective dose groups of table 3. As indicated before (*2*), the relationship of median log latent period in days to log dose, in ml., of plasma containing the virus of erythroblastosis is linear and of constant slope. The present results reveal an exception, inasmuch as it is plain that the data obtained with the dose 10^{-1} ml. of plasma are not linear with the values observed with the other doses. With this dose, the deviation is considerable toward a

relatively short latent period. It is probable that this unusual response was related to the potency of the virus preparation employed, which was more highly infectious than those previously studied. This can be seen by comparison of the latent periods following the 10^{-1} ml. dose in this experiment with those observed for comparable volumes of plasma in the earlier studies [table 1 of (*2*)]. Since the data obtained with the other doses do conform to the relations previously established, straight lines were constructed through these points. Those obtained with the 10^{-1} ml. dose have been indicated separately in the graph to show the deviations observed. The latter data were not included in these further analyses.



TEXT-FIGURE 3.—The relations of median log latent period in days to log dose of the virus of erythroblastosis inoculated intravenously in chicks of various ages in the same experiment. The lines were constructed graphically with the slope constant for these relations. The points not joined by the lines are those described in the text as deviating from the usual relations seen with this agent.

The relationships of text-figure 3 clearly show a trend toward increasingly rapid response of the chick hosts to this virus with increase in age within the limits of the study. As can be seen from the values of median log latent period or median latent period in days in table 3, the results showed considerable uniformity, with the exception of the reversal between the 49- and 63-day-old groups. The reason for this was entirely obscure since there was no recognizable factor in the background of these chicks to explain the discrepancy. Nevertheless, it was evident that response was

related quantitatively to the age of the host to the extent indicated in table 4, as derived from the potency ratios of the linear relations of text-figure 3. From these values, there would appear to be no question that susceptibility to the agent of erythroblastosis increases progressively with increase in age, at least to the limit of 77 days.

TABLE 4.—*Relative susceptibility of chicks of different ages to the virus of erythroblastosis given intravenously*

Age (*days*)	Average weight of chickens (*gm.*)	Relative susceptibility
7	45.3	1.0
21	181	1.8
35	272	2.6
49	577	12.9
63	812	8.8
77	998	15.3

Discussion

As in the studies (*1*) on the virus of avian myeloblastosis, there has been observed in the present work a marked dependence of host response on route of inoculation with the agent of erythroblastosis. Indeed, the findings for the two diseases were closely similar. Host susceptibility was manifested at the highest level in association with intravenous administration of the agent. Response to the intramedullary injections was slightly less, essentially identical with the difference in myeloblastosis. A still greater decrease was found with intramuscular injections, which was also in conformity with myeloblastosis. In contrast, very large differences were observed with intraperitoneal and subcutaneous injections, in comparison to both the responses to erythroblastosis given by intravenous, intramedullary, and intramuscular routes, and to the responses obtained by the same routes (intraperitoneal and subcutaneous) employed for myeloblastosis. In the latter disease, the factors of apparently higher resistance were 17.5 and 66.6, respectively, as compared with 393 and 1,400, table 2, for erythroblastosis given intraperitoneally and subcutaneously. This would appear to constitute a definite difference between the host-virus relationships which result in the induction of the two diseases.

The significance of the deviation of the pattern of response to the agent given intraperitoneally and subcutaneously from that injected intravenously is not entirely clear. In myeloblastosis it was judged that response to virus administered by these routes was essentially that to be expected from commensurately smaller quantities of agent injected intravenously. It now seems unlikely that this is the whole explanation; instead, it would appear that, although some virus given other than intravenously is sieved out and lost, the data are more fully interpreted as due in part to retarded absorption-and-presentation of the agent to the cellular elements affected in both leukemic conditions.

The outcome of the experiments with chicks of different ages was of much interest on both fundamental and practical grounds. Although it would not be justifiable to emphasize the precision of the results, it is quite clear that the susceptibility of the host chicks to infection with the virus of erythroblastosis and to the development and lethal outcome of the disease does not decrease with age to 77 days. On the contrary, there is definite evidence in the series that susceptibility to the course of the process actually increases. This response is essentially opposite that (*1*) in myeloblastosis, in which susceptibility decreases progressively with increase in age. It should be emphasized that the studies on both diseases were made in the same strain of White Leghorn chicks, the inbred line 15. Earlier, it was noted (*2*) that in their pathologic manifestations, erythroblastosis and myeloblastosis have shown no evidence of overlapping or mixture when the vehicle of experimental transmission is filtered virus, as employed in this laboratory. Dose-response is, likewise, characteristic in each condition, as are other phenomena—the low concentration of virus particles in the blood plasma and the lack of adenosinetriphosphatase activity by the micro-screening test (*7*), already reported (*2*). The present findings constitute an additional basis of distinction between these avian leukemias. The results also raise questions of fundamental interest pertaining to the nature and locus of activity of the factors concerned with resistance and susceptibility to the agent or agents of erythroblastosis and myeloblastosis. Discussion of these aspects of the problem will be postponed until more has been learned about the etiologic relationships of the conditions.

That host resistance to infection with the virus of erythroblastosis decreases with increasing age is of much practical importance for investigation of the disease. Older chicks can be employed for titration and bioassay of the agent. Birds 2 to 3 weeks of age are suitable for large-scale handling and housing and are much more easily injected intravenously than the 3-day-old ones used in myeloblastosis. Furthermore, experience has shown that dose groups of 2 or, occasionally, 3 different ages hatched at weekly intervals (total age difference 2 weeks) can be used in a single experiment by proportioning these birds according to age in the various dose groups. A great advantage is the possibility of experiments on vaccination; in myeloblastosis natural resistance increases so greatly during a vaccination period that the final test for acquired resistance would be difficult. This is not a problem in erythroblastosis. In addition, large birds can be employed as a source of plasma containing the agent of erythroblastosis rather than the very small ones which must be used in myeloblastosis.

Summary

Studies have been made on the influence of route of inoculation and host age in the dose response of chicks to the virus of erythroblastosis. Employing the response to the agent given intravenously as unity, the virus is less effective given into the medullary canal of the tibia, into the muscle, into the peritoneum, and into the subcutaneous tissues, by factors of 1.2,

22.1, 393, and 1,400. These results are qualitatively similar to those obtained in previous experiments with the virus of myeloblastosis. The experiments concerned with age were made with chicks 7, 21, 35, 49, 63, and 77 days old. There was observed a progressive *increase in susceptibility* with increase in age within the limits of the age range investigated. This was contrary to the findings in myeloblastosis, in which a progressive *decrease in susceptibility* was found with increase in age. The implications of these findings were discussed.

References

(*1*) Eckert, E. A., Beard, D., and Beard, J. W.: Dose-response relations in experimental transmission of avian erythromyeloblastic leukosis. V. Influence of host age and route of virus inoculation. J. Nat. Cancer Inst. 15: 1195–1207, 1955.

(*2*) ———: Virus of avian erythroblastosis. I. Titration of infectivity. J. Nat. Cancer Inst. 16: 1099–1120, 1956.

(*3*) Furth, J.: Erythroleukosis and the anemias of the fowl. Arch. Path. 12: 1–30, 1931.

(*4*) Engelbreth-Holm, J.: Spontaneous and Experimental Leukaemia in Animals. London, Oliver & Boyd, Ltd., 1942, pp. 245.

(*5*) Mommaerts, E. B., Sharp, D. G., Eckert, E. A., Beard, D., and Beard, J. W.: Virus of avian erythromyeloblastic leukosis. I. Relation of specific plasma particles to the dephosphorylation of adenosine triphosphate. J. Nat. Cancer Inst. 14: 1011–1025, 1954.

(*6*) Eckert, E. A., Beard, D., and Beard, J. W.: Dose-response relations in experimental transmission of avian erythromyeloblastic leukosis. III. Titration of the virus. J. Nat. Cancer Inst. 14: 1055–1066, 1954.

(*7*) Green, I., Beard, D., Eckert, E. A., and Beard, J. W.: Quantitative aspects of micro ATPase measurements on plasma from chicks with erythromyeloblastic leukosis. Proc. Soc. Exper. Biol. & Med. 85: 406–409, 1954.

(*8*) Eckert, E. A., Waters, N. F., Burmester, B. R., Beard, D., and Beard, J. W.: Dose-response relations in experimental transmission of avian erythromyeloblastic leukosis. IV. Strain differences in host-response to the virus. J. Nat. Cancer Inst. 14: 1067–1080, 1954.

(*9*) Waters, N. F.: Breeding for resistance and susceptibility to avian lymphomatosis. Poultry Sc. 24: 259–269, 1945.

(*10*) ———: Mortality from lymphomatosis and other causes among inbred lines of White Leghorns. Poultry Sc. 30: 531–545, 1951.

U. S. GOVERNMENT PRINTING OFFICE: 1958

The Journal of the National Cancer Institute is published monthly by the National Cancer Institute of the National Institutes of Health, Bethesda, Md. Twelve issues are published each year in two volumes, beginning with number 1 in July and January. The Journal publishes original observations and investigations in laboratory and clinical research in cancer. Manuscripts are considered for publication with the understanding that they have not been published previously.

Publication is not limited to members of the National Cancer Institute or to those whose work is supported by Government funds. Manuscripts should be submitted to the Journal of the National Cancer Institute, National Cancer Institute, Bethesda 14, Md.

Suggestions to Authors

1. Submit an original and two carbon copies of the manuscript. Follow the style of a recent copy of the Journal, especially for illustrations, references, and tables. The latest edition of Webster's International Dictionary is followed in matters of spelling, capitalization, punctuation, compounds, and hyphenation. Current medical dictionaries and Chemical Abstracts are usually followed for medical and chemical terms. Type material double spaced.

2. In the by-line, include the name of the institution with which each author is connected. Official title or academic degree is optional.

3. Begin each page with a new paragraph; *i.e.*, each page should contain one or more complete paragraphs, and no paragraph should carry over from one page to the next unless it is longer than a full page. Do not begin a new paragraph on a page carrying a run-over paragraph. This is a requirement of the Government Printing Office.

4. Insert each footnote in the text immediately after the line in which it is mentioned.

5. Type each table on a separate page, to be the page immediately following the paragraph in which the table is first mentioned.

6. Graphs, charts, and drawings are printed in the text and referred to as text-figures. Legends for text-figures should be included in the text immediately following the paragraph in which the text-figure is first mentioned. A drawing three times the size of that desired for the final figure is preferable. The lettering should be uniform and of such size that in the reproduction it will be approximately the same size as that of the printed type used in the text. The original drawings reproduce best and are therefore preferable. If two additional copies (photostats, etc.) of each drawing are also submitted, review of the manuscript is expedited.

7. Photographs are printed together at the end of each article, as plate pages. The maximum size for each printed plate page is 5¼ by 8 inches, and the photographs should be combined into as few plates as possible. Photographs should be submitted either in the exact size, or exactly double the size, of the final printed reproduction. Mount the photographs for each plate together on one piece of heavy cardboard. Number each photograph consecutively. Type the legends for each plate on a separate page. If, in addition, two unmounted duplicate prints of each photograph are also submitted, review of the manuscript will be expedited.

Published by the U. S. Department of Health, Education, and Welfare in accordance with section 220, title 44, U. S. Code

Approval by the Director of the Bureau of the Budget for the printing of this publication expires on 18 August 1957

UNITED STATES GOVERNMENT PRINTING OFFICE, WASHINGTON : 1956

For sale by the Superintendent of Documents, U. S. Government Printing Office Washington 25, D. C. - Price per copy $2.00.

Subscription price for two volumes per year in the United States, Canada, and Mexico, $20.00; foreign, $25.00.

CONTENTS

JOURNAL OF THE NATIONAL CANCER INSTITUTE • VOL. 17 · NO. 1 · JULY 1956

Page

Experiments on an ascites hepatoma. III. The conversion of mouse hepatomas into the ascites form. *Haruo Sato, Morris Belkin,* and *Edward Essner*.. 1

The role of graft and host vessels in the vascularization of grafts of normal and neoplastic tissue. *Ruth M. Merwin* and *Glenn H. Algire*..... 23

The influence of sex on isologous skin grafting in the mouse. *Richmond T. Prehn* and *Joan M. Main*..... 35

Quantitative studies on the multiplication of neoplastic cells *in vivo*. III. Metabolic stability of deoxypentose nucleic acid and the use of labeled tumor cells for the measurement of growth curves. *László Révész, Arne Forssberg,* and *George Klein*..... 37

A method of freeze-drying and its use in histochemistry and pathology. *M. S. Burstone*..... 49

Some biochemical differences in normal and tumor tissue (sarcoma 180) of mice: Amino acid composition. *M. N. Mickelson* and *Louis Barvick*..... 65

Recorded and expected mortality among the Navajo, with special reference to cancer. *Robert L. Smith, Clarence G. Salsbury,* and *Alexander G. Gilliam*.... 77

Virus of avian erythroblastosis. II. Influence of host age and route of inoculation on dose-response. *G. S. Beaudreau, R. A. Bonar, D. Beard,* and *J. W. Beard*..... 91